PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/00, 15/61, 9/90, 15/85, 15/86, 5/10, 5/20, C07K 16/40, C12Q 1/68, A01K 67/027, G01N 33/50 — A1

(11) International Publication Number: WO 97/24435

(43) International Publication Date: 10 July 1997 (10.07.97)

(21) International Application Number: PCT/US96/20785

(22) International Filing Date: 30 December 1996 (30.12.96)

(30) Priority Data:

60/009,409	29 December 1995 (29.12.95)	US
08/580,539	29 December 1995 (29.12.95)	US
60/010,835	30 January 1996 (30.01.96)	US
08/594,242	30 January 1996 (30.01.96)	US
08/632,175	12 April 1996 (12.04.96)	US

(71) Applicant: DARWIN MOLECULAR CORPORATION [US/US]; 1631-220th Street Southeast, Bothell, WA 98021 (US).

(71)(72) Applicant and Inventor: OSHIMA, Junko [US/US]; 5200 Ravenna Avenue N.E., Seattle, WA 98105 (US).

(72) Inventors: FU, Ying-Hui; 7417-28th Avenue N.W., Seattle, WA 98117 (US). YU, Chang-En; 5709-60th Avenue N.E., Seattle, WA 98105 (US). MULLIGAN, John; 5823-17th Avenue Northeast, Seattle, WA 98105 (US). SCHELLENBERG, Gerald, D.; 7031-19th Avenue Northwest, Seattle, WA 98117 (US).

(74) Agents: NOTTENBURG, Carol, Ph., D. et al.; Seed and Berry L.L.P., 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).

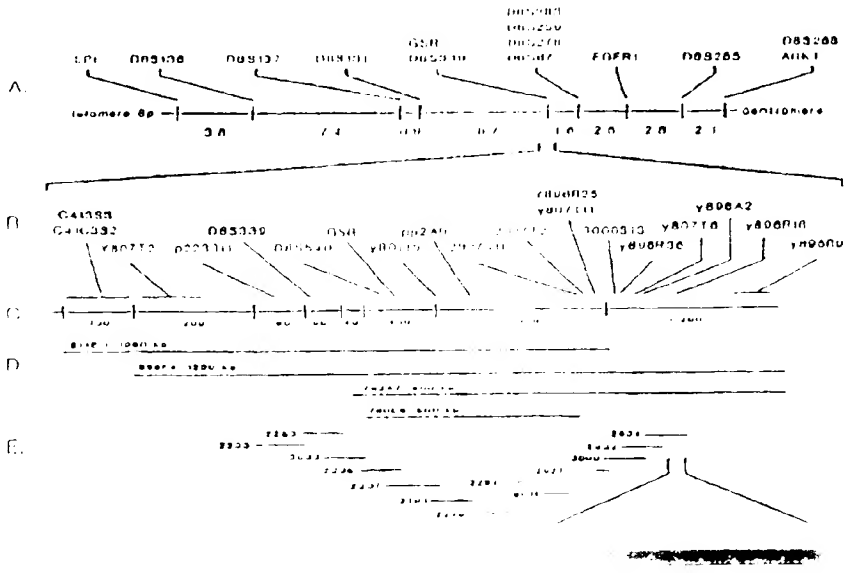
(81) Designated States: AL, AM, AT, AU, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GENES AND GENE PRODUCTS RELATED TO WERNER'S SYNDROME



(57) Abstract

The present invention discloses nucleic acid molecules encoding WRN gene products, expression vectors and host cells suitable for expressing such products.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	[illegible]
[illegible]	[illegible]				
[illegible]	[illegible] Republic		[illegible]		[illegible]
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	[illegible] States [illegible]
FR	[illegible]	**MN**	[illegible]	[illegible]	Uzbekistan
[illegible]	[illegible]	**MR**	[illegible]	[illegible]	[illegible]

DESCRIPTION

GENES AND GENE PRODUCTS RELATED TO WERNER'S SYNDROME

TECHNICAL FIELD

The present invention relates generally to Werner's Syndrome and more specifically to methods and compositions suitable for use in diagnosis and treatment of Werner's Syndrome.

BACKGROUND OF THE INVENTION

Werner Syndrome (WS) is an autosomal recessive disorder with a complex phenotype. The disorder manifests itself in premature occurrence of age-related diseases and premature appearance of some of the physical features of normal aging. The onset of symptoms usually occurs after adolescence. The disorder progresses throughout life and typically patients have a shortened life expectancy with a modal age of death at 47. The prevalence of Werner Syndrome is estimated for heterozygotes to be 1-5 per 1,000 individuals, and for homozygotes to be 1-22 per 1,000,000 individuals.

Clinical symptoms of Werner Syndrome include both a prevalence of age-related diseases and physical features of aging. Such diseases include arteriosclerosis and heart disease, both benign and malignant neoplasms (usually sarcomas), diabetes mellitus, osteoporosis, and ocular cataracts. The physical appearance of WS patients is often manifest as a short stature, premature graying or loss of hair, hypogonadism, altered skin pigmentation, hyperkeratosis, tight skin, bird-like facies, cutaneous atrophy, cutaneous leg ulcers, and telangiectasia. Most of these diseases and features are present in from 40-90% of WS patients. Diagnosis of WS relies mainly upon the appearance of a certain number of these diseases and features

In addition to the noted signs and symptoms of aging, Werner Syndrome mimics normal aging as evidenced by the replicative potential of fibroblasts isolated from WS subjects. Replication potential of fibroblasts is reduced in these patients compared to fibroblasts isolated from age-matched controls, and is comparable to the replicative potential of fibroblasts taken from elderly subjects. Moreover, an increased mutation rate has been described in WS patients. Such abnormality is manifest as chromosomal instability, such as inversions, reciprocal translocations, deletions, and pseudodiploidy, and as increased mutation rate at the hypoxanthine phosphoribosyl transferase (HPRT) gene.

Werner Syndrome has been recognized as an autosomal recessive disorder. Goto et al. (Goto et al., *Nature 355*:735-738, 1992) mapped the WS gene onto the short arm of chromosome 8, using 21 affected Japanese families. The gene is located between marker D8S87 and ankyrin (ANK1). More recently, more refined mapping has pinpointed the WS gene to a region between marker D8S131 and D8S87, an 8.3 cM interval. Identification of the gene and gene product should add considerably to understanding the basis of Werner Syndrome and enable biochemical and genetic approaches to diagnosis and treatment.

The present invention provides a novel, previously unidentified gene for Werner Syndrome and compositions for diagnosis and treatment of WS, and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides isolated nucleic acid molecules encoding the WRN gene, as well as portions thereof, representative of which are provided in the Figures. The protein which is encoded by the WRN gene is referred to hereinafter as the "WRN protein". Within other embodiments, nucleic acid molecules are provided which encode a mutant WRN gene product that increases the

Within other aspects of the present invention, isolated nucleic acid molecules are provided, selected from the group consisting of (a) an isolated nucleic acid molecule as set forth in the Figures, or complementary sequence thereof, (b) an isolated nucleic acid molecule that specifically hybridizes to the nucleic acid molecule of (a) under conditions of high stringency, and (c) an isolated nucleic acid that encodes a WRN gene product (WRN protein). As utilized herein, it should be understood that a nucleic acid molecule hybridizes "specifically" to an WRN gene (or related sequence) if it hybridizes detectably to such a sequence, but does not significantly or detectably hybridize to the Bloom's Syndrome gene (Ellis et al., *Cell 83*:655-666, 1995).

Within other aspects, expression vectors are provided comprising a promoter operably linked to one of the nucleic acid molecule described above. Representative examples of suitable promoters include tissue-specific promoters, as well as promoters such as the CMV I-E promoter, SV40 early promoter and MuLV LTR. Within related aspects, viral vectors are provided that are capable of directing the expression of a nucleic acid molecule as described above. Representative examples of such viral vectors include herpes simplex viral vectors, adenoviral vectors, adenovirus-associated viral vectors and retroviral vectors. Also provided are host cells (*e.g.*, human, dog, monkey, rat or mouse cells) which carry the above-described vectors.

Within other aspects of the present invention, isolated proteins or polypeptides are provided comprising a WRN gene product, as well as peptides of greater than 12, 13 or 20 amino acids. Within another embodiment, the protein is a mutant WRN gene product that increases the probability of Werner's Syndrome.

Within yet another aspect of the present invention, methods of treating or preventing Werner's Syndrome are provided (as well as for related diseases which are discussed in more detail below), comprising the step of administering to a patient a vector containing or expressing a nucleic acid molecule as described above, thereby reducing the likelihood or delaying the onset of Werner's Syndrome (or the related

administering to a patient a protein as described above, thereby reducing the likelihood

or delaying the onset of Werner's Syndrome (or a related disease) in the patient. Within certain embodiments, the above methods may be accomplished by *in vivo* administration.

Also provided by the present invention are pharmaceutical compositions comprising a nucleic acid molecule, vector, host cell, protein, or antibody as described above, along with a pharmaceutically acceptable carrier or diluent.

Within other aspects of the present invention, antibodies are provided which specifically bind to an WRN protein or to unique peptides derived therefrom. As utilized herein, it should be understood that an antibody is specific for an WRN protein (or peptide) if it binds detectably, and with a K_d of 10^{-7}M or less (*e.g.*, 10^{-8}M, 10^{-9}M, etc.), but does not bind detectably (or with an affinity of greater than 10^{-7}M, (*e.g.*, 10^{-6}M, 10^{-5}M, etc.) to an unrelated helicase (*e.g.*, the Bloom's Syndrome gene, *supra*). Also provided are hybridomas which are capable of producing such antibodies.

Within other aspects of the present invention, nucleic acid probes are provided which are capable of specifically hybridizing (as defined below) to an WRN gene under conditions of high stringency. Within one related aspect, such probes comprise at least a portion of the nucleotide sequence shown in the Figures, or its complementary sequence, the probe being capable of specifically hybridizing to a mutant WRN gene under conditions of high stringency. Representative probes of the present invention are generally at least 12 nucleotide bases in length, although they may be 14, 16, 18 bases or longer. Also provided are primer pairs capable of specifically amplifying all or a portion of any of the nucleic acid molecules disclosed herein.

Within other aspects of the invention, methods are provided for diagnosing a patient having an increased likelihood of contracting Werner's Syndrome (or a related disease), comprising the steps of (a) obtaining from a patient a biological sample containing nucleic acid, (b) incubating the nucleic acid with a probe which is capable of specifically hybridizing to a mutant WRN gene under condition[illegible]

[illegible] determining that said patient [illegible]

of contracting Werner's Syndrome (or a related disease). Within another aspect,

methods are provided comprising the steps of (a) obtaining from a patient a biological sample containing nucleic acid, (b) amplifying a selected nucleic acid sequence associated with a mutant WRN gene, and (c) detecting the presence of an amplified nucleic acid sequence, and thereby determining that the patient has an increased likelihood of contracting Werner's Syndrome (or a related disease). Suitable biological samples include nucleated cells obtained from the peripheral blood, from buccal swabs, or brain tissue.

Within another aspect, peptide vaccines are provided which comprise a portion of a mutant WRN gene product containing a mutation, in combination with a pharmaceutically acceptable carrier or diluent.

Within yet another aspect, transgenic animals are provided whose germ cells and somatic cells contain a WRN gene (or lack thereof, *i.e.*, a "knockout") which is operably linked to a promoter effective for the expression of the gene, the gene being introduced into the animal, or an ancestor of the animal, at an embryonic stage. Within one embodiment, the animal is a mouse, rat or dog. Within other embodiments, the WRN gene is expressed from a vector as described above. Within yet another embodiment, the WRN gene encodes a mutant WRN gene product.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTING

Figure 1 is a genetic and physical map of the WRN region. The genetic map (A) of the region is sex-equal with distances given in cM. The polymorphic loci used (B) are di-nucleotide and tri-nucleotide repeat STRP loci. The physical map

2253, 3833, 2236, and 3101. Marker order was determined from the sequence-tagged

site (STS) content of YACs, P1 clones, and cosmid clones and from genomic DNA sequence from P1 clones. The YACs presented (D) represent the minimal tiling and are the YACs used for cDNA selection experiments. The P1 and cosmid clones needed for the minimum tiling path are shown (E). Clones shown are P1 clones except for 8C11, which is a cosmid clone. Clone order was established by STS content.

Figures 2A and 2B are the DNA (SEQ ID No. 70) and predicted amino acid (SEQ ID No. 71) sequences of the WRN gene transcript. The one-letter amino acid code is used in Fig. 2B.

Figures 3A-3C are the DNA and predicted amino acid sequence of an alternate WRN gene transcript (SEQ ID Nos. 72 and 73).

Figures 4A-4G are an alignment of the WRN gene product (SEQ ID No. 74) with known helicases from *S. pombe* (SEQ ID No. 76), *E. coli* (SEQ ID No. 75), human (SEQ ID No. 77) and the Bloom's Syndrome gene "BLM" (SEQ ID No. 78).

Figures 5A-5U are the genomic DNA sequence of the region containing a WRN gene (SEQ ID No. 79).

Figure 6 presents a cDNA sequence of the mouse WRN gene (SEQ ID Nos. 205 and 206).

Figure 7 is a genomic DNA sequence of the mouse WRN gene (SEQ ID Nos. 207-209).

Figure 8 is a diagram of the WRN gene product with location of mutations. A. WRN cDNA. Numbering across the top refers to the cDNA sequence as numbered in GenBank L76937. B. Predicted WRN gene product. The helicase domain is designated as "HD", motifs from I to VI are indicated. C. Location of mutations. Numbering across the bottom refer to the mutations. *: nonsense mutation. ^: frame shift mutation caused by a single base deletion. Gray lines: frame shift mutations causing deletion of exon(s). D. Predicted proteins. Lines represent the different predicted truncated proteins produced from mutations in the WRN.

WRN gene product by [illegible] staining sparse [illegible] panel B, and the size of cells (panel C) expressing the WRN gene.

Figure 10 shows the alignment of the mouse and human WRN gene products.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Prior to setting forth the invention in detail, it may be helpful to an understanding thereof to set forth definitions of certain terms and to list and to define the abbreviations that will be used hereinafter.

"Genetic marker" is any segment of a chromosome that is distinguishably unique in the genome, and polymorphic in the population so as to provide information about the inheritance of linked DNA sequences, genes and/or other markers.

"Vector" refers to an assembly which is capable of directing the expression of a WRN gene, as well as any additional sequence(s) or gene(s) of interest. The vector must include transcriptional promoter elements which are operably linked to the genes of interest. The vector may be composed of either deoxyribonucleic acids ("DNA"), ribonucleic acids ("RNA"), or a combination of the two (*e.g.*, a DNA-RNA chimeric). Optionally, the vector may include a polyadenylation sequence, one or more restriction sites, as well as one or more selectable markers such as neomycin phosphotransferase or hygromycin phosphotransferase. Additionally, depending on the host cell chosen and the vector employed, other genetic elements such as an origin of replication, additional nucleic acid restriction sites, enhancers, sequences conferring inducibility of transcription, and selectable markers, may also be incorporated into the vectors described herein.

Abbreviations: YAC, yeast artificial chromosome; EST, expressed sequence tag; PCR, polymerase chain reaction; RT-PCR, PCR process in which RNA is first transcribed into DNA at the first step using reverse transcriptase (RT); cDNA, any

compositions for the detection and treatment of Werner's Syndrome, as well as related

diseases. These methods and compositions include a family of Werner's Syndrome-related genes, and the proteins encoded thereby, that have been implicated in the onset of Werner's Syndrome. These genes and proteins, including genetic markers, nucleic acid sequences and clones, are also useful in the creation of *in vitro* and animal models and screening tests useful for the study of Werner's Syndrome, including the possible identification of other genes implicated in Werner's Syndrome. The present invention also provides vector constructs, genetic markers, nucleic acid sequences, clones, diagnostic tests and compositions and methods for the identification of individuals likely to suffer from Werner's Syndrome.

Genes and Gene Products Related To Werner's Syndrome

The present invention provides isolated nucleic acid molecules comprising a portion of the gene which is implicated in the onset of WS. Briefly, as can be seen from Figure 4, this gene encodes a protein that is similar in amino acid sequence to several known ATP-dependent DNA helicases (enzymes that unwind the DNA duplex). It is less similar to known RNA-DNA helicases. Helicases are involved in the replication of DNA, often binding the replication origin, and/or the replication complex. In addition, the single stranded DNA that is involved in recombination can be generated by DNA helicases.

Although various aspects of the WRN gene (or portions thereof) are shown in the Figures, it should be understood that within the context of the present invention, reference to one or more of these genes includes derivatives of the genes that are substantially similar to the genes (and, where appropriate, the proteins (including peptides and polypeptides) that are encoded by the genes and their derivatives). As used herein, a nucleotide sequence is deemed to be "substantially similar" if: (a) the nucleotide sequence is derived from the coding region of the described genes and includes, for example, portions of the sequence or allelic variations of the sequences

hybridization to nucleotide sequences of the present invention under high or very high

stringency (*see* Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, NY, 1989); or (c) the nucleic acid sequences are degenerate as a result of the genetic code to the nucleic acid sequences defined in (a) or (b). Further, the nucleic acid molecule disclosed herein includes both complementary and non-complementary sequences, provided the sequences otherwise meet the criteria set forth herein. Within the context of the present invention, high stringency means standard hybridization conditions (*e.g.*, 5x SSPE, 0.5% SDS at 65°C, or the equivalent) while very high stringency means conditions of hybridization such that the nucleotide sequence is able to selectively hybridize to a single allele of the WS-related gene.

The WRN gene may be isolated from genomic DNA or cDNA. Genomic DNA libraries constructed in chromosomal vectors, such as YACs (yeast artificial chromosomes), bacteriophage vectors, such as λEMBL3, λgt10, cosmids, or plasmids are suitable for use. cDNA libraries constructed in bacteriophage vectors, plasmids, or others, are suitable for screening. Such libraries may be constructed using methods and techniques known in the art (*see* Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989) or purchased from commercial sources (*e.g.*, Clontech, Palo Alto, CA). Within one embodiment, the WRN gene is isolated by PCR performed on genomic DNA, cDNA or DNA from libraries, or is isolated by probe hybridization of genomic DNA or cDNA libraries. Primers for PCR and probes for hybridization screening may be designed based on the DNA sequence of WRN presented herein. The DNA sequence of a portion of the WRN gene and the entire coding sequence is presented in the Figures. Primers for PCR should be derived from sequences in the 5' and 3' untranslated region in order to isolate a full-length cDNA. The primers should not have self-complementary sequences nor have complementary sequences at their 3' end (to prevent primer-dimer formation). Preferably, the primers have a GC content of about 50% and contain restriction sites. The primers are annealed to cDNA and sufficient cycles of PCR are performed to yield

[illegible] oligonucleotide hybridization probe suitable for screening genomic or

cDNA libraries may be designed based on the sequence provided herein. Preferably, the oligonucleotide is 20-30 bases long. Such an oligonucleotide may be synthesized by automated synthesis. The oligonucleotide may be conveniently labeled at the 5' end with a reporter molecule, such as a radionuclide, (*e.g.*, ^{32}P) or biotin. The library is plated as colonies or phage, depending upon the vector, and the recombinant DNA is transferred to nylon or nitrocellulose membranes. Following denaturation, neutralization, and fixation of the DNA to the membrane, the membranes are hybridized with the labeled probe. The membranes are washed and the reporter molecule detected. The hybridizing colonies or phage are isolated and propagated. Candidate clones or PCR amplified fragments may be verified as containing WRN DNA by any of various means. For example, the candidate clones may be hybridized with a second, nonoverlapping probe or subjected to DNA sequence analysis. In these ways, clones containing WRN gene, which are suitable for use in the present invention are isolated.

The structure of the proteins encoded by the nucleic acid molecules described herein may be predicted from the primary translation products using the hydrophobicity plot function of, for example, P/C Gene, Lasergen System, DNA STAR, Madison, Wisconsin, or according to the methods described by Kyte and Doolittle (*J. Mol. Biol. 157*:105-132, 1982).

WRN proteins of the present invention may be prepared in the form of acidic or basic salts, or in neutral form. In addition, individual amino acid residues may be modified by oxidation or reduction. Furthermore, various substitutions, deletions, or additions may be made to the amino acid or nucleic acid sequences, the net effect of which is to retain or further enhance or decrease the biological activity of the mutant or wild-type protein. Moreover, due to degeneracy in the genetic code, for example, there may be considerable variation in nucleotide sequences encoding the same amino acid sequence.

Other derivatives of the WRN proteins disclosed herein include

which may be added to facilitate purification or identification of WRN proteins (see

U.S. Patent No. 4,851,341; *see also*, Hopp et al., *Bio/Technology* 6:1204, 1988.) Alternatively, fusion proteins such as WRN protein-β-galactosidase or WRN protein-luciferase may be constructed in order to assist in the identification, expression, and analysis of WRN proteins.

WRN proteins of the present invention may be constructed using a wide variety of techniques described herein. Further, mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes a derivative having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific (or segment specific) mutagenesis procedures may be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); and Sambrook et al. (*supra*). Deletion or truncation derivatives of WRN proteins (*e.g.*, a soluble extracellular portion) may also be constructed by utilizing convenient restriction endonuclease sites adjacent to the desired deletion. Subsequent to restriction, overhangs may be filled in, and the DNA religated. Exemplary methods of making the alterations set forth above are disclosed by Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, 1989).

Mutations of the present invention preferably preserve the reading frame of the coding sequences. Furthermore, the mutations will preferably not create complementary regions that could hybridize to produce secondary mRNA structures, such as loops or hairpins, that would adversely affect translation of the mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the

target codon and the expressed mutants screened for indicative biological activity

Alternatively, mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes a derivative having the desired amino acid insertion, substitution, or deletion.

WRN proteins may also be constructed utilizing techniques of PCR mutagenesis, chemical mutagenesis (Drinkwater and Klinedinst, *PNAS 83*:3402-3406, 1986), by forced nucleotide misincorporation (*e.g.*, Liao and Wise *Gene 88*:107-111, 1990), or by use of randomly mutagenized oligonucleotides (Horwitz et al., *Genome 3*:112-117, 1989).

Proteins can be isolated by, among other methods, culturing suitable host and vector systems to produce the recombinant translation products of the present invention. Supernates from such cell lines, or protein inclusions or whole cells where the protein is not excreted into the supernate, can then be treated by a variety of purification procedures in order to isolate the desired proteins. For example, the supernate may be first concentrated using commercially available protein concentration filters, such as an Amicon or Millipore Pellicon ultrafiltration unit. Following concentration, the concentrate may be applied to a suitable purification matrix such as, for example, an anti-protein antibody bound to a suitable support. Alternatively, anion or cation exchange resins may be employed in order to purify the protein. As a further alternative, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps may be employed to further purify the protein. Other methods of isolating the proteins of the present invention are well known in the skill of the art.

A protein is deemed to be "isolated" within the context of the present invention if no other (undesired) protein is detected pursuant to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis followed by Coomassie blue staining. Within other embodiments, the desired protein can be isolated such that no

Expression of a WRN gene

The present invention also provides for the manipulation and expression of the above described genes by culturing host cells containing a vector capable of expressing the above-described genes. Such vectors or vector constructs include either synthetic or cDNA-derived nucleic acid molecules encoding WRN proteins, which are operably linked to suitable transcriptional or translational regulatory elements. Suitable regulatory elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, insect, or plant genes. Selection of appropriate regulatory elements is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of regulatory elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a transcriptional terminator, and a ribosomal binding sequence, including a translation initiation signal.

Nucleic acid molecules that encode any of the WRN proteins described above may be readily expressed by a wide variety of prokaryotic and eukaryotic host cells, including bacterial, mammalian, yeast or other fungi, viral, insect, or plant cells. Methods for transforming or transfecting such cells to express foreign DNA are well known in the art (see, *e.g.*, Itakura et al., U.S. Patent No. 4,704,362; Hinnen et al., *Proc. Natl. Acad. Sci. USA 75*:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950; Axel et al., U.S. Patent No. 4,399,216; Goeddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, 1989; for plant cells see Czako and Marton, *Plant Physiol 104*:1067-1071, 1994; and Paszkowski et al., *Biotech. 24*:387-392, 1992).

Bacterial host cells suitable for carrying out the present invention include *E. coli*, *B. subtilis*, *Salmonella typhimurium*, and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, as well as many other bacterial species well known to one of ordinary skill in the art. Representative examples of

[illegible] functions in the host cell, one or more selectable phenotypic markers, and a bacterial

origin of replication. Representative promoters include the β-lactamase (penicillinase) and lactose promoter system (*see* Chang et al., *Nature 275*:615, 1978), the T7 RNA polymerase promoter (Studier et al., *Meth. Enzymol. 185*:60-89, 1990), the lambda promoter (Elvin et al., *Gene 87*:123-126, 1990), the *trp* promoter (Nichols and Yanofsky, *Meth. in Enzymology 101*:155, 1983) and the *tac* promoter (Russell et al., *Gene 20*: 231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Many plasmids suitable for transforming host cells are well known in the art, including among others, pBR322 (*see* Bolivar et al., *Gene 2*:95, 1977), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (*see* Messing, *Meth. in Enzymology 101*:20-77, 1983 and Vieira and Messing, *Gene 19*:259-268, 1982), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.).

Yeast and fungi host cells suitable for carrying out the present invention include, among others, *Saccharomyces pombe*, *Saccharomyces cerevisiae*, the genera *Pichia* or *Kluyveromyces* and various species of the genus *Aspergillus* (McKnight et al., U.S. Patent No. 4,935,349). Suitable expression vectors for yeast and fungi include, among others, YCp50 (ATCC No. 37419) for yeast, and the amdS cloning vector pV3 (Turnbull, *Bio/Technology 7*:169, 1989), YRp7 (Struhl et al., *Proc. Natl. Acad. Sci. USA 76*:1035-1039, 1978), YEp13 (Broach et al., *Gene 8*:121-133, 1979), pJDB249 and pJDB219 (Beggs, *Nature 275*:104-108, 1978) and derivatives thereof.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., *J. Biol. Chem. 255*:12073-12080, 1980; Alber and Kawasaki, *J. Mol. Appl. Genet. 1*:419-434, 1982) or alcohol dehydrogenase genes (Young et al., in *Genetic Engineering of Microorganisms for Chemicals*, Hollaender et al. (eds.), p. 355, Plenum, New York, 1982; Ammerer, *Meth. Enzymol. 101*:192-201, 1983). Examples of useful promoters for fungi vectors include those derived from *Aspergillus nidulans* glycolytic genes, such as the *adh3* promoter (McKnight et al.

et al., 1985).

As with bacterial vectors, the yeast vectors will generally include a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include *leu2* (Broach et al., *ibid.*), *ura3* (Botstein et al., *Gene 8*:17, 1979), or *his3* (Struhl et al., *ibid.*). Another suitable selectable marker is the *cat* gene, which confers chloramphenicol resistance on yeast cells.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (*ibid.*), Hinnen et al. (*Proc. Natl. Acad. Sci. USA 75*:1929-1933, 1978), Yelton et al. (*Proc. Natl. Acad. Sci. USA 81*:1740-1747, 1984), and Russell (*Nature 301*:167-169, 1983). The genotype of the host cell may contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

Protocols for the transformation of yeast are also well known to those of ordinary skill in the art. For example, transformation may be readily accomplished either by preparation of spheroplasts of yeast with DNA (*see* Hinnen et al., *PNAS USA* 75:1929, 1978) or by treatment with alkaline salts such as LiCl (*see* Itoh et al., *J Bacteriology 153*:163, 1983). Transformation of fungi may also be carried out using polyethylene glycol as described by Cullen et al. (*Bio/Technology 5*:369, 1987).

Viral vectors include those which comprise a promoter that directs the expression of an isolated nucleic acid molecule that encodes an WRN protein as described above. A wide variety of promoters may be utilized within the context of the present invention, including for example, promoters such as MoMLV LTR, RSV LTR, Friend MuLV LTR, adenoviral promoter (Ohno et al., *Science 265*: 781-784, 1994), neomycin phosphotransferase promoter/enhancer, late parvovirus promoter (Koering

[illegible] promoter, and the cytomegalovirus immediate late promoter. Within particularly

preferred embodiments of the invention, the promoter is a tissue-specific promoter (see *e.g.*, WO 91/02805; EP 0,415,731; and WO 90/07936). Representative examples of suitable tissue specific promoters include neural specific enolase promoter, platelet derived growth factor beta promoter, bone morpho-genetic protein promoter, human alpha1-chimaerin promoter, synapsin I promoter and synapsin II promoter. In addition to the above-noted promoters, other viral-specific promoters (*e.g.*, retroviral promoters (including those noted above, as well as others such as HIV promoters), hepatitis, herpes (*e.g.*, EBV), and bacterial, fungal or parasitic (*e.g.*, malarial) -specific promoters may be utilized in order to target a specific cell or tissue which is infected with a virus, bacteria, fungus or parasite.

Thus, WRN proteins of the present invention may be expressed from a variety of viral vectors, including for example, herpes viral vectors (*e.g.*, U.S. Patent No. 5,288,641), adenoviral vectors (*e.g.*, WO 94/26914, WO 93/9191; Kolls et al., *PNAS 91*(1):215-219, 1994; Kass-Eisler et al., *PNAS 90*(24):11498-502, 1993; Guzman et al., *Circulation 88*(6):2838-48, 1993; Guzman et al., *Cir. Res. 73*(6):1202-1207, 1993; Zabner et al., *Cell 75*(2):207-216, 1993; Li et al., *Hum Gene Ther. 4*(4):403-409, 1993; Caillaud et al., *Eur. J. Neurosci. 5*(10:1287-1291, 1993; Vincent et al., *Nat. Genet. 5*(2):130-134, 1993; Jaffe et al., *Nat. Genet. 1*(5):372-378, 1992; and Levrero et al, *Gene 101*(2):195-202, 1991), adeno-associated viral vectors (WO 95/13365; Flotte et al., *PNAS 90*(22):10613-10617, 1993), baculovirus vectors, parvovirus vectors (Koering et al., *Hum. Gene Therap. 5*:457-463, 1994), pox virus vectors (Panicali and Paoletti, *PNAS 79*:4927-4931, 1982; and Ozaki et al., *Biochem. Biophys. Res. Comm. 193*(2):653-660, 1993), and retroviruses (*e.g.*, EP 0,415,731; WO 90/07936; WO 91/0285, WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218. Viral vectors may likewise be constructed which contain a mixture of different elements (*e.g.*, promoters, envelope sequences and the like) from different viruses, or non-viral sources. Within various embodiments, either the viral

Mammalian cells suitable for carrying out the present invention include, among others: PC12 (ATCC No. CRL1721), N1E-115 neuroblastoma, SK-N-BE(2)C neuroblastoma, SHSY5 adrenergic neuroblastoma, NS20Y and NG108-15 murine cholinergic cell lines, or rat F2 dorsal root ganglion line, COS (*e.g.*, ATCC No. CRL 1650 or 1651), BHK (*e.g.*, ATCC No. CRL 6281; BHK 570 cell line (deposited with the American Type Culture Collection under accession number CRL 10314), CHO (ATCC No. CCL 61), HeLa (*e.g.*, ATCC No. CCL 2), 293 (ATCC No. 1573; Graham et al., *J. Gen. Virol. 36*:59-72, 1977) and NS-1 cells. Other mammalian cell lines may be used within the present invention, including Rat Hep I (ATCC No. CRL 1600), Rat Hep II (ATCC No. CRL 1548), TCMK (ATCC No. CCL 139), Human lung (ATCC No. CCL 75.1), Human hepatoma (ATCC No. HTB-52), Hep G2 (ATCC No. HB 8065), Mouse liver (ATCC No. CCL 29.1), NCTC 1469 (ATCC No. CCL 9.1), SP2/0-Ag14 (ATCC No. 1581), HIT-T15 (ATCC No. CRL 1777), and RINm 5AHT$_2$B (Orskov and Nielson, *FEBS 229*(1):175-178, 1988).

Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Viral promoters include the cytomegalovirus immediate early promoter (Boshart et al., *Cell 41*:521-530, 1985), cytomegalovirus immediate late promoter, SV40 promoter (Subramani et al., *Mol Cell. Biol. 1*:854-864, 1981), MMTV LTR, RSV LTR, metallothionein-1, adenovirus E1a. Cellular promoters include the mouse metallothioncin-1 promoter (Palmiter et al., U.S. Patent No. 4,579,821), a mouse V_K promoter (Bergman et al., *Proc. Natl. Acad. Sci. USA 81*:7041-7045, 1983; Grant et al., *Nucl. Acids Res. 15*:5496, 1987) and a mouse V_H promoter (Loh et al., *Cell 33*:85-93, 1983). The choice of promoter will depend, at least in part, upon the level of expression desired or the recipient cell line to be transfected.

Such expression vectors may also contain a set of RNA splice sites

adenovirus and or immunoglobulin genes. Also contained in the expression vectors is a

polyadenylation signal located downstream of the coding sequence of interest. Suitable polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, *ibid.*), the polyadenylation signal from the Adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., *Nuc. Acids Res.* 9:3719-3730, 1981). The expression vectors may include a noncoding viral leader sequence, such as the Adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer. Expression vectors may also include sequences encoding the adenovirus VA RNAs. Suitable expression vectors can be obtained from commercial sources (*e.g.*, Stratagene, La Jolla, Calif.).

Vector constructs comprising cloned DNA sequences can be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., *Cell 14*:725, 1978; Corsaro and Pearson, *Somatic Cell Genetics 7*:603, 1981; Graham and Van der Eb, *Virology 52*:456, 1973), electroporation (Neumann et al., *EMBO J. 1*:841-845, 1982), or DEAE-dextran mediated transfection (Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., NY, 1987). To identify cells that have stably integrated the cloned DNA, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. Preferred amplifiable selectable markers are the DHFR gene and the neomycin resistance gene. Selectable markers are reviewed by Thilly (*Mammalian Cell Technology*, Butterworth Publishers, Stoneham, MA, which is incorporated herein by reference).

Mammalian cells containing a suitable vector are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the

[illegible] manner to select for increased copy number of the cloned sequences, thereby increasing

expression levels. Cells expressing the introduced sequences are selected and screened for production of the protein of interest in the desired form or at the desired level. Cells that satisfy these criteria can then be cloned and scaled up for production.

Protocols for the transfection of mammalian cells are well known to those of ordinary skill in the art. Representative methods include calcium phosphate mediated transfection, electroporation, lipofection, retroviral, adenoviral and protoplast fusion-mediated transfection (*see* Sambrook et al., *supra*). Naked vector constructs can also be taken up by muscular cells or other suitable cells subsequent to injection into the muscle of a mammal (or other animals).

Numerous insect host cells known in the art can also be useful within the present invention, in light of the subject specification. For example, the use of baculoviruses as vectors for expressing heterologous DNA sequences in insect cells has been reviewed by Atkinson et al. (*Pestic. Sci. 28*:215-224,1990).

Numerous plant host cells known in the art can also be useful within the present invention, in light of the subject specification. For example, the use of *Agrobacterium rhizogenes* as vectors for expressing genes in plant cells has been reviewed by Sinkar et al., (*J. Biosci. (Bangalore) 11*:47-58, 1987).

WRN proteins may be prepared by growing (typically by culturing) the host/vector systems described above, in order to express the recombinant WRN proteins. Recombinantly produced WRN proteins may be further purified as described in more detail below.

Within related aspects of the present invention, WRN proteins may be expressed in a transgenic animal whose germ cells and somatic cells contain a WRN gene which is operably linked to a promoter effective for the expression of the gene. Alternatively, in a similar manner transgenic animals may be prepared that lack the WRN gene (*e.g.*, "knockout" mice). Such transgenics may be prepared in a variety non-human animals, including mice, rats, rabbits, sheep, dogs, goats and pigs (*see* Hammer

41:343-345, 1985 and U.S. Patent Nos. 5,175,383, 5,087,571, 4,736,866, 5,387,742, 5,347,075, 5,221,778, and 5,175,384).

Briefly, an expression vector, including a nucleic acid molecule to be expressed together with appropriately positioned expression control sequences, is introduced into pronuclei of fertilized eggs, for example, by microinjection. Integration of the injected DNA is detected by blot analysis of DNA from tissue samples. It is preferred that the introduced DNA be incorporated into the germ line of the animal so that it is passed on to the animal's progeny. Tissue-specific expression may be achieved through the use of a tissue-specific promoter, or through the use of an inducible promoter, such as the metallothionein gene promoter (Palmiter et al., 1983, *ibid*), which allows regulated expression of the transgene.

Vectors of the present invention may contain or express a wide variety of additional nucleic acid molecules in place of or in addition to an WRN protein as described above, either from one or several separate promoters. For example, the viral vector may express a lymphokine or lymphokine receptor, antisense or ribozyme sequence or toxins. Representative examples of lymphokines include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF, G-CSF, M-CSF, alpha-interferon, beta-interferon, gamma-interferon, and tumor necrosis factors, as well as their respective receptors. Representative examples of antisense sequences include antisense sequences which block the expression of WRN protein mutants. Representative examples of toxins include: ricin, abrin, diphtheria toxin, cholera toxin, saporin, gelonin, pokeweed antiviral protein, tritin, *Shigella* toxin, and *Pseudomonas* exotoxin A.

Within other aspects of the invention, antisense oligonucleotide molecules are provided which specifically inhibit expression of mutant WRN nucleic acid sequences (*see generally*, Hirashima et al. in *Molecular Biology of RNA: New Perspectives* (M. Inouye and B. S. Dudock, eds., 1987 Academic Press, San Diego, p.

[illegible] U.S. 5,359,051; WO 92/06693; and EP-A2-0512844). Briefly, such

molecules are constructed such that they are complementary to, and able to form Watson-Crick base pairs with, a region of transcribed WRN mutant mRNA sequence containing an WRN mutation. The resultant double-stranded nucleic acid interferes with subsequent processing of the mRNA, thereby preventing protein synthesis.

Within other related aspects of the invention, ribozyme molecules are provided wherein an antisense oligonucleotide sequence is incorporated into a ribozyme which can specifically cleave mRNA molecules transcribed from a mutant WRN gene (*see generally*, Kim et al. *Proc Nat. Acad. Sci. USA 84*:8788 (1987); Haseloff, et al. *Nature 234*:585 (1988), Cech, *JAMA 260*:3030 (1988); Jeffries, et al. *Nucleic Acids Res. 17*:1371 (1989); U.S. 5,093,246; U.S. 5,354,855, U.S. 5,144,019; U.S. 5,272,262; U.S. 5,254,678; and U.S. 4,987,071). According to this aspect of the invention, the antisense sequence which is incorporated into a ribozyme includes a sequence complementary to, and able to form Watson-Crick base pairs with, a region of the transcribed mutant WRN mRNA containing an WRN mutation. The antisense sequence thus becomes a targeting agent for delivery of catalytic ribozyme activity specifically to mutant WRN mRNA, where such catalytic activity cleaves the mRNA to render it incapable of being subsequently processed for WRN protein translation.

Host Cells

As discussed above, nucleic acid molecules which encode the WRN proteins of the present invention (or the vectors which contain and/or express related mutants) may readily be introduced into a wide variety of host cells. Representative examples of such host cells include plant cells, eukaryotic cells, and prokaryotic cells. Within preferred embodiments, the nucleic acid molecules are introduced into cells from a vertebrate or warm-blooded animal, such as a human, macaque, dog, cow, horse, pig, sheep, rat, hamster, mouse or fish cell, or any hybrid thereof.

Preferred prokaryotic host cells for use within the present invention

[illegible] cloned therein are well known in the art (see, e.g., Maniatis et al., *Molecular*

Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, which is incorporated herein by reference; or Sambrook et al., *supra*). Vectors used for expressing cloned DNA sequences in bacterial hosts will generally contain a selectable marker, such as a gene for antibiotic resistance, and a promoter that functions in the host cell. Appropriate promoters include the *trp* (Nichols and Yanofsky, *Meth. Enzymol. 101*:155-164, 1983), *lac* (Casadaban et al., *J. Bacteriol. 143*:971-980, 1980), and phage λ (Queen, *J. Mol. Appl. Genet. 2*:1-10, 1983) promoter systems. Plasmids useful for transforming bacteria include the pUC plasmids (Messing, *Meth. Enzymol. 101*:20-78, 1983; Vieira and Messing, *Gene 19*:259 268, 1982), pBR322 (Bolivar et al., *Gene 2*:95-113, 1977), pCQV2 (Queen, *ibid.*), and derivatives thereof. Plasmids may contain both viral and bacterial elements.

Preferred eukaryotic cells include cultured mammalian cell lines (*e.g.*, rodent or human cell lines) and fungal cells, including species of yeast (*e.g.*, *Saccharomyces* spp., particularly *S. cerevisiae*, *Schizosaccharomyces* spp., or *Kluyveromyces* spp.) or filamentous fungi (*e.g.*, *Aspergillus* spp., *Neurospora* spp.). Strains of the yeast *Saccharomyces cerevisiae* are particularly preferred. Methods for producing recombinant proteins in a variety of prokaryotic and eukaryotic host cells are generally known in the art (*see*, "Gene Expression Technology," *Methods in Enzymology*, Vol. 185, Goeddel (ed.), Academic Press, San Diego, Calif., 1990; *see also*, "Guide to Yeast Genetics and Molecular Biology," *Methods in Enzymology*, Guthrie and Fink (eds.), Academic Press, San Diego, Calif., 1991). In general, a host cell will be selected on the basis of its ability to produce the protein of interest at a high level or its ability to carry out at least some of the processing steps necessary for the biological activity of the protein. In this way, the number of cloned DNA sequences that must be introduced into the host cell can be minimized and overall yield of biologically active protein can be maximized.

The nucleic acid molecules (or vectors) may be introduced into host cells

Pearson, *Somatic Cell Gen.* 7:603, 1981; Graham and Van der Eb, *Virology* 52:456,

1973), electroporation (Neumann et al., *EMBO J.* 1:841-845, 1982), retroviral, adenoviral, protoplast fusion-mediated transfection or DEAE-dextran mediated transfection (Ausubel et al., (eds.), *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., NY, NY, 1987).

Host cells containing vector constructs of the present invention are then cultured to express a DNA molecule as described above. The cells are cultured according to standard methods in a culture medium containing nutrients required for growth of the chosen host cells. A variety of suitable media are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals, as well as other components, *e.g.*, growth factors or serum, that may be required by the particular host cells. The growth medium will generally select for cells containing the DNA construct(s) by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct.

Suitable growth conditions for yeast cells, for example, include culturing in a chemically defined medium, comprising a nitrogen source, which may be a non-amino acid nitrogen source or a yeast extract, inorganic salts, vitamins and essential amino acid supplements at a temperature between 4°C and 37°C, with 30°C being particularly preferred. The pH of the medium is preferably maintained at a pH greater than 2 and less than 8, more preferably pH 5-6. Methods for maintaining a stable pH include buffering and constant pH control. Preferred agents for pH control include sodium hydroxide. Preferred buffering agents include succinic acid and Bis-Tris (Sigma Chemical Co., St. Louis, Mo.). Due to the tendency of yeast host cells to hyperglycosylate heterologous proteins, it may be preferable to express the nucleic acid molecules of the present invention in yeast cells having a defect in a gene required for asparagine-linked glycosylation. Such cells are preferably grown in a medium containing an osmotic stabilizer. A preferred osmotic stabilizer is sorbitol

Cultured mammalian cells are generally cultured in commercially available serum-containing or serum-free media. Selection of a medium and growth conditions appropriate for the particular cell line used is well within the level of ordinary skill in the art.

Antibodies

Antibodies to the WRN proteins discussed above may readily be prepared given the disclosure provided herein. Such antibodies may, within certain embodiments, specifically recognize wild type WRN protein rather than a mutant WRN protein, mutant WRN protein rather than wild type WRN protein, or equally recognize both the mutant and wild-type forms of WRN protein. Antibodies may be used for isolation of the protein, establishing intracellular localization of the WRN protein, inhibiting activity of the protein (antagonist), or enhancing activity of the protein (agonist). Knowledge of the intracellular location of the WRN gene product may be abnormal in patients with WRN mutations, thus allowing the development of a rapid screening assay. As well, assays for small molecules that interact with the WRN gene product will be facilitated by the development of antibodies and localization studies.

Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, anti-idiotypic antibodies, antibody fragments (*e.g.*, Fab, and $F(ab')_2$, F_V variable regions, or complementarity determining regions). As discussed above, antibodies are understood to be specific against an WRN protein if it binds with a K_d of greater than or equal to 10^{-7}M, preferably greater than of equal to 10^{-8}M. The affinity of a monoclonal antibody or binding partner can be readily determined by one of ordinary skill in the art (*see* Scatchard, *Ann. N.Y. Acad. Sci. 51*:660-672, 1949).

Briefly, polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, [illegible] cross-linking with glutaraldehyde) is utilized to immunize the animal, through

intraperitoneal, intramuscular, intraocular, or subcutaneous injections, an adjuvant such as Freund's complete or incomplete adjuvant. Merely as an example, a peptide corresponding to residues 1375 through 1387 of the WRN polypeptide sequence is used to raise a rabbit polyclonal antiserum. Following several booster immunizations, samples of serum are collected and tested for reactivity to the WRN protein or peptide. Particularly preferred polyclonal antisera will give a signal on one of these assays that is at least three times greater than background. Once the titer of the animal has reached a plateau in terms of its reactivity to the protein, larger quantities of antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

Monoclonal antibodies may also be readily generated using conventional techniques (*see* U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993 which are incorporated herein by reference; *see also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference).

Briefly, within one embodiment a subject animal such as a rat or mouse is injected with an WRN protein or portion thereof as described above. The protein may be admixed with an adjuvant such as Freund's complete or incomplete adjuvant in order to increase the resultant immune response. Between one and three weeks after the initial immunization the animal may be reimmunized with another booster immunization, and tested for reactivity to the protein utilizing assays described above. Once the animal has reached a plateau in its reactivity to the injected protein, it is sacrificed, and organs which contain large numbers of B cells such as the spleen and lymph nodes are harvested.

Cells which are obtained from the immunized animal may be immortalized by transfection with a virus such as the Epstein-Barr virus (EBV) (*see*

suitable myeloma cell in order to create a "hybridoma" which secretes monoclonal

antibody. Suitable myeloma lines include, for example, NS-1 (ATCC No. TIB 18), and P3X63 - Ag 8.653 (ATCC No. CRL 1580).

Following the fusion, the cells may be placed into culture plates containing a suitable medium, such as RPMI 1640, or DMEM (Dulbecco's Modified Eagles Medium) (JRH Biosciences, Lenexa, Kansas), as well as additional ingredients, such as fetal bovine serum (FBS, *i.e.*, from Hyclone, Logan, Utah, or JRH Biosciences). Additionally, the medium should contain a reagent which selectively allows for the growth of fused spleen and myeloma cells such as HAT (hypoxanthine, aminopterin, and thymidine) (Sigma Chemical Co., St. Louis, Missouri). After about seven days, the resulting fused cells or hybridomas may be screened in order to determine the presence of antibodies which are reactive against an WRN protein. A wide variety of assays may be utilized to determine the presence of antibodies which are reactive against the proteins of the present invention, including for example countercurrent immuno-electrophoresis, radioimmunoassays, radioimmunoprecipitations, enzyme-linked immuno-sorbent assays (ELISA), dot blot assays, western blots, immunoprecipitation, Inhibition or Competition Assays, and sandwich assays (*see* U.S. Patent Nos. 4,376,110 and 4,486,530; *see also Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Following several clonal dilutions and reassays, a hybridoma producing antibodies reactive against the WRN protein may be isolated.

Other techniques may also be utilized to construct monoclonal antibodies (*see* William D. Huse et al., "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," *Science 246*:1275-1281, December 1989; *see also* L. Sastry et al., "Cloning of the Immunological Repertoire in *Escherichia coli* for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific cDNA Library," *Proc. Natl. Acad. Sci. USA 86*:5728-5732, August 1989; *see also* Michelle Alting-Mees et al., "Monoclonal Antibody Expression

La Jolla, California, which enables the production of antibodies through recombinant

techniques). Briefly, mRNA is isolated from a B cell population, and utilized to create heavy and light chain immunoglobulin cDNA expression libraries in the λ ImmunoZap(H) and λImmunoZap(L) vectors. These vectors may be screened individually or co-expressed to form Fab fragments or antibodies (*see* Huse et al., *supra*; *see also* Sastry et al., *supra*). Positive plaques may subsequently be converted to a non-lytic plasmid which allows high level expression of monoclonal antibody fragments from *E. coli*.

Similarly, portions or fragments, such as Fab and Fv fragments, of antibodies may also be constructed utilizing conventional enzymatic digestion or recombinant DNA techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody. Within one embodiment, the genes which encode the variable region from a hybridoma producing a monoclonal antibody of interest are amplified using nucleotide primers for the variable region. These primers may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. Stratacyte (La Jolla, Calif.) sells primers for mouse and human variable regions including, among others, primers for V_{Ha}, V_{Hb}, V_{Hc}, V_{Hd}, C_{H1}, V_L and C_L regions. These primers may be utilized to amplify heavy or light chain variable regions, which may then be inserted into vectors such as ImmunoZAP™ H or ImmunoZAP™ L (Stratacyte), respectively. These vectors may then be introduced into *E. coli*, yeast, or mammalian-based systems for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the V_H and V_L domains may be produced (*see* Bird et al., *Science 242*:423-426, 1988). In addition, such techniques may be utilized to change a "murine" antibody to a "human" antibody, without altering the binding specificity of the antibody.

Once suitable antibodies have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (*see Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor

combination of these techniques.

Assays

Assays useful within the context of the present invention include those assays for detecting agonists or antagonists of WRN protein activity. Other assays are useful for the screening of peptide or organic molecule libraries. Still other assays are useful for the identification and/or isolation of nucleic acid molecules and/or peptides within the present invention, the identification of proteins that interact or bind the WRN protein, for diagnosis of a patient with an increased likelihood of contracting Werner's Syndrome, or for diagnosis of a patient with susceptibility to or manifestation of a WRN-related disease.

Nucleic Acid Based Diagnostic Tests

Briefly, another aspect of the present invention provides probes and primers for detecting the WRN genes and/or mutants thereof. In one embodiment of this aspect, probes are provided that are capable of specifically hybridizing to DNA or RNA of the WRN genes. For purposes of the present invention, probes are "capable of hybridizing" to DNA or RNA of the WRN gene if they hybridize to an WRN gene under conditions of either high or moderate stringency (*see* Sambrook et al., *supra*) but not significantly or detectably to the an unrelated helicase gene such as the Bloom's Syndrome gene (Ellis et al., *Cell 83*:655-666, 1995). Preferably, the probe hybridizes to suitable nucleotide sequences under high stringency conditions, such as hybridization in 5x SSPE, 1x Denhardt's solution, 0.1% SDS at 65°C, and at least one wash to remove unhybridized probe in the presence of 0.2x SSC, 1x Denhardt's solution, 0.1% SDS at 65°C. Except as otherwise provided herein, probe sequences are designed to allow hybridization to WRN genes, but not to DNA or RNA sequences from other genes. The probes are used, for example, to hybridize to nucleic acid that is present in a biological sample isolated from a patient. The hybridized probe is then detected, thereby [illegible] hybridization. Alternatively, the WRN gene may be amplified and the amplified

product subjected to DNA sequencing. Mutants of WRN may be detected by DNA sequence analysis or hybridization with allele-specific oligonucleotide probes under conditions and for time sufficient to allow hybridization to the specific allele. Typically, the hybridization buffer and wash will contain tetramethyl ammonium chloride or the like (*see* Sambrook et al., *supra*).

Nucleic acid probes of the present invention may be composed of either deoxyribonucleic acids (DNA), ribonucleic acids (RNA), nucleic acid analogues (*e.g.*, peptide nucleic acids), or any combination thereof, and may be as few as about 12 nucleotides in length, usually about 14 to 18 nucleotides in length, and possibly as large as the entire sequence of a WRN gene. Selection of probe size is somewhat dependent upon the use of the probe, and is within the skill of the art.

Suitable probes can be constructed and labeled using techniques that are well known in the art. Shorter probes of, for example, 12 bases can be generated synthetically and labeled with ^{32}P using T_4 polynucleotide kinase. Longer probes of about 75 bases to less than 1.5 kb are preferably generated by, for example, PCR amplification in the presence of labeled precursors such as [α-^{32}P]dCTP, digoxigenin-dUTP, or biotin-dATP. Probes of more than 1.5 kb are generally most easily amplified by transfecting a cell with a plasmid containing the relevant probe, growing the transfected cell into large quantities, and purifying the relevant sequence from the transfected cells. (*See* Sambrook et al., *supra*.)

Probes can be labeled by a variety of markers, including for example, radioactive markers, fluorescent markers, enzymatic markers, and chromogenic markers. The use of ^{32}P is particularly preferred for marking or labeling a particular probe.

It is a feature of this aspect of the invention that the probes can be utilized to detect the presence of WRN mRNA or DNA within a sample. However, if the relevant sample is present in only a limited number, then it may be beneficial to

equence including, for example, RNA amplification (see Lizardi et al.,

Bio/Technology 6:1197-1202, 1988; Kramer et al., *Nature* 339:401-402, 1989; Lomeli et al., *Clinical Chem.* 35(9):1826-1831, 1989; U.S. Patent No. 4,786,600), and DNA amplification utilizing LCR or polymerase chain reaction ("PCR") (*see*, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159) (*see also* U.S. Patent Nos. 4,876,187 and 5,011,769, which describe an alternative detection/amplification system comprising the use of scissile linkages), or other nucleic acid amplification procedures that are well within the level of ordinary skill in the art. With respect to PCR, for example, the method may be modified as known in the art. Transcriptional enhancement of PCR may be accomplished by incorporation of bacteriophage T7 RNA polymerase promoter sequences in one of the primary oligonucleotides, and immunoenzymatic detection of the products from the enhanced emitter may be effected using anti-RNA:DNA antibodies (Blais, *Appl. Environ. Microbiol.* 60:348-352, 1994). PCR may also be used in combination with reverse dot-blot hybridization (Iida et al., *FEMS Microbiol. Lett.* 114:167-172, 1993). PCR products may be quantitatively analyzed by incorporation of dUTP (Duplàa et al., *Anal. Biochem.* 212:229-236, 1993), and samples may be filter sampled for PCR-gene probe detection (Bej et al., *Appl. Environ. Microbiol.* 57:3529-3534, 1991).

Within a particularly preferred embodiment, PCR amplification is utilized to detect the WRN DNA. Briefly, as described in greater detail below, a DNA sample is denatured at 95°C in order to generate single-stranded DNA. The DNA sample may be a cDNA generated from RNA. Specific primers are then annealed to the single-stranded DNA at 37°C to 70°C, depending on the proportion of AT/GC in the primers. The primers are extended at 72°C with *Taq* DNA polymerase or other thermostable DNA polymerase in order to generate the opposite strand to the template. These steps constitute one cycle, which may be repeated in order to amplify the selected sequence. For greater specificity, nested PCR may be performed. In nested PCR, a second amplification is performed using a second set of primers derived from sequences

convenient size for determining their sequence. In a preferred embodiment, nested PCR is performed.

Within an alternative preferred embodiment, LCR amplification is utilized for amplification. LCR primers are synthesized such that the 5' base of the upstream primer is capable of hybridizing to a unique base pair in a desired gene to specifically detect an WRN gene.

Within another preferred embodiment, the probes are used in an automated, non-isotopic strategy wherein target nucleic acid sequences are amplified by PCR, and then desired products are determined by a colorimetric oligonucleotide ligation assay (OLA) (Nickerson et al., *Proc. Natl. Acad. Sci. USA 87*:8923-8927, 1990).

Primers for the amplification of a selected sequence should be selected from sequences that are highly specific to WRN (and not, *e.g.*, the Bloom's Syndrome gene, *supra*) and form stable duplexes with the target sequence. The primers should also be non-complementary, especially at the 3' end, should not form dimers with themselves or other primers, and should not form secondary structures or duplexes with other regions of DNA. In general, primers of about 18 to 20 nucleotides are preferred, and can be easily synthesized using techniques well known in the art. PCR products, and other nucleic acid amplification products, may be quantitated using techniques known in the art (Duplàa et al., *Anal. Biochem. 212*:229-236, 1993; Higuchi et al., *Bio/Technology 11*:1026-1030).

Within one embodiment of the invention, nucleic acid diagnostics may be developed which are capable of detecting the presence of Werner's Syndrome, or of various related diseases that may be caused by Werner's Syndrome. Briefly, severe mutations in the WRN gene may lead to Werner's Syndrome, as well as a host of related diseases, including for example, increased frequency of some benign and malignant neoplasms (especially sarcomas), cataracts, cardiovascular disease, osteoporosis, type I

[illegible] In addition, many of the related diseases may be associated with mutations in

the WRN gene. For example, diabetes and osteoporosis are often associated with aging. Aging population and individuals with these (or other) diseases are screened for mutations in WRN. Any of the assays described herein may be used. RT-PCR is especially preferred in conjunction with DNA sequence determination. To correlate a mutation or polymorphism with disease, sibling pairs in which one sibling has disease are preferred subjects. Once a mutation is identified, other convenient screening assays may be used to assay particular nucleotide changes.

Since the sequences of the two copies of the gene from non-Werner's affected individuals can be correlated with the medical histories of these patients to define these correspondences, these alleles can therefore be used as diagnostics for susceptibilities to these diseases, once the relationship is defined. Certain non-null forms of the gene, for example, in either the homozygous or heterozygous state may significantly affect the propensity for the carriers to develop, for example, cancer. These propensities can be ascertained by examining the sequences of the gene (both copies) in a statistically significant sample of cancer patients. Other diseases (see above) can be similarly examined for significant correlations with certain alleles. To detect such a causal relationship one can use a chi-squared test, or other statistical test, to examine the significance of any correlation between the appropriate genotypes and the disease state as recorded in the medical records, using standard good practices of medical epidemiology. The sequences that define each of the alleles are then valuable diagnostic indicators for an increased susceptibility to the disease. Thus, from the nucleic acid sequences provided herein, a wide variety of Werner's Syndrome-related diseases may be readily detected.

Another cellular phenotype of the cells from Werner's patients is the increased frequency of deletion mutation in these cells. Clearly, the defective helicase in these cells leads to a specific mutator phenotype, while not rendering the cells hypersensitive to a variety of chemical or physical mutagens that damage DNA, like

some alleles may therefore be diagnostic of this class of medical conditions.

Assays for agonists and antagonists

Also provided by the present invention are agonists or antagonists of the WRN gene product comprising a protein, peptide, chemical, or peptidomimetic that binds to the WRN gene product or interacts with a protein that binds to the WRN gene product such that the binding of the agonist or antagonist affects the activity of the WRN gene product. An agonist will activate or increase the activity of the WRN gene product. An antagonist will inhibit or decrease the activity of the WRN gene product. The activity of the WRN gene product may be measured in an assay, such as a helicase assay or other assay that measures an activity of the WRN gene product. Other assays measure the binding of protein that interacts with WRN and is necessary for its activity.

Agonists and antagonists of the WRN gene product may be used to enhance activity or inhibit activity of the gene product. Such agonists and antagonists may be identified by a variety of methods. For example, proteins that bind and activate WRN may be identified using a yeast 2 hybrid detection system. In this system, the WRN gene is fused to either a DNA-binding domain or an activating domain of a yeast gene such as GAL4. A cDNA library is constructed in a vector such that the inserts are fused to one of the domains. The vectors are co-transfected into yeast and selected for transcriptional activation of a reporter gene (Fields and Song, *Nature 340*: 245, 1989). The protein(s) that bind to WRN are candidate agonists. Three different proteins that bind WRN have been identified in an initial screen using the 2-hybrid system.

When the binding site on WRN gene product is determined, molecules that bind and activate WRN protein can be designed and evaluated. For example, computer modeling of the binding site can be generated and mimetics that bind can be designed. Antibodies to the binding site may be generated and analogues of native binding proteins generated as well. Any of these molecules is tested for agonist or antagonist activity by a functional assay of the WRN gene product. For example, to test

administered and activation is monitored. An antagonist will inhibit the activation of

the reporter gene by at least 50%. Similarly, agonist activity may be measured by either enhancing WRN activity in a yeast 2-hybrid system or by coupling the test compound to a DNA binding or activation domain and monitoring activity of the reporter gene.

Labels

WRN proteins, nucleic acid molecules which encodes such proteins, anti-WRN protein antibodies and agonists or antagonists, as described above and below, may be labeled with a variety of molecules, including for example, fluorescent molecules, toxins, and radionuclides. Representative examples of fluorescent molecules include fluorescein, *Phycobili* proteins, such as phycoerythrin, rhodamine, Texas red and luciferase. Representative examples of toxins include ricin, abrin diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin, *Shigella* toxin, and *Pseudomonas* exotoxin A. Representative examples of radionuclides include Cu-64, Ga-67, Ga-68, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I-131, Re-186, Re-188, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212. In addition, the antibodies described above may also be labeled or conjugated to one partner of a ligand binding pair. Representative examples include avidin-biotin, and riboflavin-riboflavin binding protein.

Methods for conjugating or labeling the WRN proteins, nucleic acid molecules which encode such proteins, anti-WRN protein antibodies and agonists or antagonists, as discussed above, with the representative labels set forth above may be readily accomplished by one of ordinary skill in the art (*see* Trichothecene Antibody Conjugate, U.S. Patent No. 4,744,981,; Antibody Conjugate, U.S. Patent No. 5,106,951; Fluorogenic Materials and Labeling Techniques, U.S. Patent No. 4,018,884; Metal Radionuclide Labeled Proteins for Diagnosis and Therapy, U.S. Patent No. 4,897,255; and Metal Radionuclide Chelating Compounds for Improved Chelation Kinetics, U.S Patent No. 4,988,496; *see also* Inman, *Methods In Enzymology* Vol. 34 *Affinity* [illegible] Bioanalytical Applications," *Anal. Biochem.* 174:1-32, 1988).

Pharmaceutical Compositions

As noted above, the present invention also provides a variety of pharmaceutical compositions, comprising one of the above-described WRN proteins, nucleic acid molecules, vectors, antibodies, host cells, agonists or antagonists, along with a pharmaceutically or physiologically acceptable carrier, excipients or diluents. Generally, such carriers should be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the therapeutic agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents.

In addition, the pharmaceutical compositions of the present invention may be prepared for administration by a variety of different routes. In addition, pharmaceutical compositions of the present invention may be placed within containers, along with packaging material which provides instructions regarding the use of such pharmaceutical compositions. Generally, such instructions will include a tangible expression describing the reagent concentration, as well as within certain embodiments, relative amounts of excipient ingredients or diluents (*e.g.*, water, saline or PBS) which may be necessary to reconstitute the pharmaceutical composition.

Methods of Treating or Preventing Werner's Syndrome

The present invention also provides methods for treating or preventing Werner's Syndrome (or related diseases), comprising the step of administering to a patient a vector (*e.g.*, expression vector, viral vector, or viral particle containing a vector) or nucleic acid molecules alone, as described above, thereby reducing the

may be used to delay onset of Werner's Syndrome, lessen symptoms, or halt or delay

progression of the disease. Such therapeutics may be tested in a transgenic animal model that expresses mutant protein, wild-type and mutant protein, or in an *in vitro* assay system (*e.g.*, a helicase assay such as that described by Bjornson et al., *Biochem. 3307*:14306-14316, 1994).

As noted above, the present invention provides methods for treating or preventing Werner's Syndrome through the administration to a patient of a therapeutically effective amount of an antagonist or pharmaceutical composition as described herein. Such patients may be identified through clinical diagnosis based on the classical symptoms of Werner's Syndrome.

As will be evident to one of skill in the art, the amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth. Typically, the compositions may be administered by a variety of techniques, as noted above.

Within other embodiments of the invention, the vectors which contain or express the nucleic acid molecules which encode the WRN proteins described above, or even the nucleic acid molecules themselves may be administered by a variety of alternative techniques, including for example administration of asialoosomucoid (ASOR) conjugated with poly-L-lysine DNA complexes (Cristano et al., *PNAS* 92122-92126, 1993), DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther.* 3(2):147-154, 1992), cytofectin-mediated introduction (DMRIE-DOPE, Vical, Calif.), direct DNA injection (Acsadi et al., *Nature 352*:815-818, 1991); DNA ligand (Wu et al., *J. of Biol. Chem. 264*:16985-16987, 1989); lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989); liposomes (Pickering et al., *Circ. 89*(1):13-21, 1994; and Wang et al., *PNAS 84*:7851-7855, 1987); microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991); and direct delivery of nucleic acids which encode the WRN protein itself either alone (Vile and Hart, *Cancer Res.* 53:3860-3864, 1993) or

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

CLONING OF THE WRN GENE FROM CHROMOSOME 8

The WS locus (*WRN*) was initially localized to 8p12 by conventional mapping methods (Goto et al., *Nature 355*:735-738, 1992) and the genetic position refined using both meiotic and homozygosity mapping (Schellenberg *et al.*, 1992; Nakura, et al., *Genomics 23*:600-608, 1994; Thomas, *Genomics 16*:685-690, 1993). The latter approach is possible since many WS subjects are the offspring of consanguineous marriages (Table 1). Initial mapping work (Nakura, et al., *Genomics 23*:600-608, 1994; Oshima et al., *Genomics 23*:100-113, 1994) placed the WRN locus in an 8.3 cM interval flanked by *D8S137* and *D8S87* (Fig. 1). *D8S339*, a marker within this interval, was the closest locus tested ($q = 0.001$, $Z_{max} = 15.93$). Multipoint analysis placed WRN within 0.6 cM of *D8S339*, although the region between *D8S87* and *FGFR* could not be excluded. Subsequently, the short tandem repeat polymorphism (STRP) markers at glutathione reductase (*GSR*) and *D8S339* were found to be in linkage disequilibrium with WS in Japanese WS subjects (Yu, *American Journal of Human Genetics 55*:356-364, 1994).

To clone the WRN gene, a yeast artificial chromosome (YAC) P1, and cosmid contig was generated starting at the *GSR/D8S339* region and extended by walking methods to cover approximately 3 Mb. An additional 16 STRP markers in the YAC contig (Fig. 1B) were identified to define recombinants and to delineate the boundaries of the linkage disequilibrium region. For marker ordering and gene identification, cosmids and P1 clones were also isolated and used to construct a small-clone partial contig of the region (Fig. 1E). The WRN region was defined by obligate recombinants at C41C3S3 excluding the region telomeric to this marker, and at 896R9

region

Genes in the WRN region were identified by exon trapping using vector pSL3 (Buckler et al., *Proc. Natl. Acad. Sci. USA 88*:4005-4009, 1991; Church et al., *Nat. Genet. 6*:98-105, 1994), hybridization of cDNA libraries to immobilized YACs (Parimoo et al., *Proc. Natl. Acad. Sci USA 87*:3166-3169, 1991), and comparison of the genomic sequence to DNA sequence databases using BLAST (Altschul et al. *J. Mol. Biol. 215*:403-410, 1990) and the exon-finding program GRAIL (Uberbacher and Mural, *Proc. Natl. Acad. Sci. USA 88*:1261, 1991). The genomic sequence was determined for the region defined by P1 clones 2233, 2253, 3833, 2236, 2237, 2932, 6738 and 2934 and cosmid clone 176 C6. Each method identifies short segments of expressed sequences, which were then used to screen an arrayed fibroblast cDNA library to identify longer cDNA clones. This library was selected because WS fibroblasts have a premature senescence phenotype *in vitro*, indicating that the WRN gene is probably expressed in this cell type. Genes identified by this process were screened for WRN mutations using reverse transcriptase-polymerase chain reaction (RT-PCR). Seven subjects were initially screened for mutations: 5 WRN subjects (2 Caucasians and 3 Japanese) and 2 control subjects (1 Caucasian and 1 Japanese). Prior to identification of the WRN gene, the following genes from the region were screened for mutations; GSR, PP2AB, TFIIEB, and genes corresponding to other expressed sequence tagged sites (ESTs).

The candidate WRN locus gene was initially detected by using the genomic sequence of P1 clone 2934 to search the EST database. A single 245 bp EST, R58879, was detected which is homologous to 3 segments of the genomic sequence separated by presumed intronic sequence. Sequence from R58879 was used to identify longer cDNA clones from a normal fibroblast cDNA library. An initial 2.1 kb cDNA clone containing EST R58879, which corresponds to the 3' end of the gene, was obtained by screening an array of clones by PCR, using the primers A and B (see below). Primers A and B are derived from R58879 sequence and yield a 145 bp

located in P2934 and [illegible] sequences contained in the initial 2.1 kb clone. Six additional

clones were identified. An additional 8 clones were obtained by plaque hybridization. The longest clone is 4.0 kb in length. Additional sequence was obtained by the RAGE method using primer 5EA to prime first strand cDNA synthesis. A 2.5 kb product was obtained that contained an additional 1.4 kb of sequence.

Evidence that R58879 is expressed was obtained by Northern blot analysis, in which 6.5 kb and 8 kb transcripts were detected in a variety of tissues, including heart, placenta, muscle, and pancreas. Also, transcripts were detected by RT-PCR products from fibroblast and lymphoblastoid cell line RNA.

EXAMPLE 2

CLONING OF THE WRN GENE FROM SUBJECTS

The WRN gene may be isolated from patients and mutations or polymorphisms determined by sequence analysis. Peripheral blood cells are obtained by venipuncture and hypotonic lysis of erythrocytes. DNA or RNA is isolated from these cells and the WRN gene isolated by amplification. The gene sequence may be obtained by amplification of the exons from genomic DNA or by RT-PCR, followed by determination of the DNA sequence. Primers suitable for determining the DNA sequence and for performing RT-PCR are listed below (Primers A-R are SEQ ID Nos. 1-18 respectively, and primers 5EA-5EG are SEQ ID Nos. 19-25 respectively). Two cDNAs were identified and are shown in Figures 2 and 3. There is some uncertainty regarding the identity of a few bases in the 5' untranslated region in Figure 2

Two RT-PCR reactions are used to obtain the gene from different tissues. First strand cDNA synthesis is carried out according to standard procedures (*e.g.*, with a Stratascript Kit from Stratagene). The cDNA is subjected to a pair of nested PCR amplifications, the first with primers I and J (SEQ ID Nos. 9 and 10), followed by primers K and L (SEQ ID Nos. 11 and 12), and the second with primers

[illegible] the gene sequence or splicing pattern. Primers A-H (SEQ ID Nos. 1-8)and K-R (SEQ

ID Nos. 11-18) are used for sequencing the first RT-PCR fragment. Primers B, 5EA, 5EB, 5EC, 5EE, 5EF and 5EG (SEQ ID Nos. 2, 19, 20, 21, 23, 24, and 25, repectively) are used for sequencing the second RT-PCR fragment. Sequencing is done on an ABI373A using Applied Biosystems Division of Perkin-Elmer FS sequencing kits according to the instructions of the manufacturer.

A	5'-CTGGCAAGGATCAAACAGAGAG
B	5'-CTTTATGAAGCCAATTTCTACCC
C	5'-TGGCAAATTGGTAGAAGCTAGG
D	5'-AAATAACTATGCTTTCTTACATTTAC
F	5'-CTCCCGTCAACTCAGATATGAG
F	5'-CTGTTTGTAAATGTAAGAAAGCATAG
G	5'-GAGCTATGATGACACCACTGC
H	5'-ACTGAGCAACAGAGTGAGACC
I	5'-GGATCTGGTCTCACTCTGTTGC
J	5'-TTGCCTAGTGCAATTGGTCTCC
K	5'-AGTGCAGTGGTGTCATCATAGC
L	5'-CCTATTTAATGGCACCCAAAATGC
M	5'-CAGTCTATGGCCATCACATACTC
N	5'-ACCGCTTGGGATAAGTGCATGC
O	5'-GAGAAGAAGTCTAACTTGGAGAAG
P	5'-TTCTGGTGACTGTACCATGATAC
Q	5'-CCAAAGGAAGTGATACCAGCAAG
R	5'-ACAGCAAGAAACATAATTGTTCTGG
5EA	5'-GAACTTTGAAGTCCATCACGACC
5EB	5'-GCATTAATAAAGCTGACATTCGCC
5EC	5'-CATTACGGTGCTCCTAAGGACATG
5ED	5'-GATGGATTTGAAGATGGAGTAGAAG
5EE	5'-TGAAAGACAATATGGAAAGAGCTTG
5EF	5'-GTAGAACCAACTCATTCTAAATGCT
5EG	5'-AATTTGCGTGTCATCCTTGCGCA

The exons of the 3'-end of the WRN gene can be amplified from DNA samples using the primers listed below (Primers E1A-E13B are SEQ ID Nos. 26-57, respectively). The DNA sequence is determined using the same primers and an

E1A [illegible]

	E1B	5'-CATGAAACTTGCTTCTAGGACAC
	E2A	5'-CCCAGGAGTTCGAGACCATCC
	E2B	5'-TTACAATCGGCCACATTCATCAC
	E2C	5'-TGTAATCCCAACACTTTGGGAGG
5	E2D	5'-AGTGGAAGAATTCATAGTGGATGG
	E3A	5'-TAGCTTTATGAAGCCAATTTCTACC
	E3B	5'-AATCCAAAGAATCAATAGACAAGTC
	E3C	5' GCTTGAAGGATGAGGCTCTGAG
	E3D	5'-TGTTCAGAATGAGCACGATGGG
10	E4A	5'-CTTGTGAGAGGCCTATAAACTGG
	E4B	5'-GGTAAACAGTGTAGGAGTCTGC
	E5A	5'-GCCATTTTCTCTTTAATTGGAAAGG
	E5B	5'-ATCTTATTCATCTTTCTGAGAATGG
	E6A	5'-TGAAATAGCCCAACATCTGACAG
15	E6B	5'-GATTAATTTGACAGCTTGATTAGGC
	E7A	5'-TGAAATATAAACTCAGACTCTTAGC
	E7B	5'-GTACTGATTTGGAAAGACATTCTC
	E8A	5'-GATGTGACAGTGGAAGCTATGG
	E8B	5' GGAAAAAATGTGGTATCTGAAGCTC
20	E9A	5'-AAGTGAGCAAATGTTGCTTCTGG
	E9B	5' TCATTAGGAAGCTGAACATCAGC
	E10A	5'-GTTGGAGGAAATTGATCCCAAGTC
	E10B	5'-TGTTGCTTATGGGTTTAACTTGTG
	E11A	5'-TAAAGGATTAATGCTGTTAACAGTG
25	E11B	5'-TCACACTGAGCATTTACTACCTG
	E12A	5' GTAATCATATCAGAATTCATAACAG
	E12B	5'-CTTTGGCAACCTTCCACCTTCC
	E12C	5'-GCAAAGGAAATGTAGCACATAGAG
	E12D	5'-AGGCTATAGGCATTTGAAAGAGG
30	E13A	5'-GTAGGCTCCCAGAAGACCCAG
	E13B	5'-GAAAGGATGGGTGTGTATTCAGG

EXAMPLE 3

IDENTIFICATION OF MUTANT ALLELES

The cDNA sequence (Figure 2) was aligned to the genomic sequence to

[illegible] patients, single base pair changes lead to splicing defects or stop codons in the open reading frame of the gene. In the fifth patient, a single base pair

change results in a cysteine to arginine transition, which may disrupt gene function. Each of the exons was also sequenced in 96 unaffected control individuals (48 Caucasians and 48 Japanese), and none of the mutations were found in any of the control individuals.

The first mutation is a mutation at a splice acceptor site. In the sequence below, the GGTAGAAA sequence begins at nucleotide 2030 (Figure 2). The g to c change results in a deletion of 95 bp.

Preparation of DNA for RT-PCR mutational analysis revealed that for one subject, the amplification product was shorter than observed in products from other WS and control subjects. DNA sequence analysis of the RT-PCR product revealed that 95 bp were missing compared to other samples. The missing sequence corresponds to a single exon. This exon and flanking genomic segments were sequenced from the WS subject and controls and a single base change (G→C) at the splice donor site was detected. The subject was the offspring of a first cousin marriage and was, as expected, homozygous for this mutation. The same mutation was found in a total of 18 out of 30 Japanese WS subjects and, thus, is the most common Japanese WS mutation. Deletion of this exon results in a change in the predicted open-reading frame and a premature stop codon. This mutation was not observed in 46 Japanese and 46 Caucasian controls. Among mutation carriers, 12/16 had the 141 bp allele at the GSR2-STRP.

wild type: ttttaatagGGTAGAAA	(SEQ ID No. 58)
Werners: ttttaatacGGTAGAAA	(SEQ ID No. 59)

The second mutation changes a C to T at nucleotide 2384 (Figure 2) changing a glutamine to a stop codon, which results in a predicted truncated protein. This mutation was observed in a single subject. Primers E11A and E11B flank this sequence and amplify a 360 bp fragment.

[illegible]

T

ter

The third mutation changes a C to T at nucleotide 2804 (Figure 2), which alters an arginine codon to a stop codon resulting in a predicted truncated protein. Four Japanese WS subjects and 1 Caucasian W5 subject had this mutation. Primers E8A and E8B flank this sequence and amplify a 267 bp product.

	arg	
wild type:	TTGGAGCGAGCA	(SEQ ID No. 62)
Werners:	TTGGAG**T**GAGCA	(SEQ ID No. 63)
	ter	

The fourth mutation is a 4 bp deletion across a splice junction. The exon sequence shown below begins at nucleotide 2579 (Figure 2). This mutation was identified in a Syrian W5 kindred. Primers E4A and E4B flank this mutation and amplify a 267 bp fragment.

wild type:	ctgt**agAC**AGACACCTC	(SEQ ID No. 68)
Werners:	ctgt----AGACACCTC	(SEQ ID No. 69)

The fifth mutation is a missense mutation. A T is altered to a G at nucleotide 2113 (Figure 2), changing the wild-type phe codon to a leu codon. This change is a polymorphism with each allele present at a frequency of approximately 0.5. It does not appear to correlate with WS.

	phe	
wild type:	AAGAAGTTTCTTCTG	(SEQ ID No. 64)
Werners:	AAGAAG**TTG**CTTCTG	(SEQ ID No. 65)
	leu	

The sixth mutation is a missense mutation changing a T to a G

wild type:	[illegible]	(SEQ ID No. 66)
Werners:	[illegible]	(SEQ ID No. 67)

arg

These point mutations may also be identified by PCR using primers that contain as the 3'-most base either the wild type or the mutant nucleotide. Two separate reactions are performed using one of these primers and a common second primer. Amplification is detectable in the reaction containing a matched primer.

EXAMPLE 4

CHARACTERIZATION OF THE WRN GENE AND GENE PRODUCT

The 2 kb WRN cDNA hybridizes to a 6.5 kb RNA and a less abundant 8 kb RNA on a Northern blot, suggesting that a full length coding region is about 5.2 kb long. An overlapping cDNA clone has been isolated that extends the sequence by 2 kb. The insert from this clone is used to probe cDNA libraries to identify other clones that contain the 5' end of the cDNA or full length sequence. Alternate splicing events are detected by sequencing the full cDNA sequence from a number of different tissues, including fully differentiated cells and stem cells, and the full range of gene transcripts identified by sequence comparison. Additional exons are identified as above by further genomic sequencing and GRAIL analysis.

The predicted amino acid sequence is shown in Figures 2B and 3. Figure 2 shows cDNA and predicted amino acid sequences of the WRN gene. Figure 3 presents cDNA and predicted amino acid sequences of a less abundant transcript of the WRN gene. The longest open reading frame is shown from the first methionine in that frame. The predicted WRN protein consists of 1,432 amino acids divided into three regions: an N-terminal region, a central region containing 7 motifs (I, Ia, II, III, IV, V and VI) characteristic of the DNA and RNA superfamily of helicases (Gorbalenya et al. *Nucleic Acid Res.* 17, 4713, 1989, [illegible]

[illegible] Because many helicases function as part of a multiprotein complex, the N-terminal

and/or the C-terminal domain may contain interaction sites for these other proteins, while the central helicase domain functions in the actual enzymatic unwinding of DNA or RNA duplexes.

The N-terminal region, encompassing approximately codons 1 to 539, is acidic; there are 109 aspartate or glutamate residues, including a stretch of 14 acidic residues in a 19 amino acid sequence (codons 507-526). Stretches of acidic residues are found in the Xeroderma pigmentosum (XP) complementation group B helicase, the Bloom's syndrome helicase, and the X-chromosome-linked α-thalassemia mental retardation syndrome helicase. In the WRN gene, this region also contains a tandem duplication of 27 amino acids in which each copy is encoded by a single exon. Because this duplication is exact at the nucleotide level, and because flanking intronic sequences for the two exons that encode the duplication are also highly similar, this duplication is presumed to be the result of a relatively recent event. The duplicated regions are also highly acidic with 8 glutamate or aspartate residues out of 27 amino acids and only 2 basic amino acids (one histidine and one lysine residue).

The central region of the WRN gene, spanning approximately codons 540-963, is highly homologous to other helicases from a wide range of organisms including the ReqQ gene from *E. coli*, the SGS1 gene from *S. cerevisiae*, a predicted helicase (F18C5C) from *C. elegans*, and several human helicases. Thus, by sequence similarity, the WRN gene is a member of a superfamily of DExH-box DNA and RNA helicases. The principle conserved sequences consist of 7 motifs found in other helicases. These motifs include a predicted nucleotide binding site (motif I) and a Mg^{2+} binding site (sequence DEAH, motif II). Some or all of the 7 motifs are presumed to form the enzymatic active site for DNA/RNA unwinding. The presence of the DEAH sequence and an ATP-binding motif further suggests that the WRN gene product is a functional helicase.

The C-terminal end of the WRN gene, from codons 964 to 1432, has

EXAMPLE 5

IDENTIFYING AND DETECTING MUTATIONS IN THE WRN GENE

Mutations or polymorphisms of WRN may be identified by various methods, including sequence analysis. Although any cell (other than erythrocytes) may be used to isolate nucleic acids, peripheral blood mononuclear cells (PBMC) are preferred. Peripheral blood mononuclear cells are obtained by venipuncture and subsequent hypotonic lysis of erythrocytes. RNA is isolated and first strand cDNA synthesis is performed using a Strata-script RT-PCR kit according to the manufacturers instructions (Stratagene, La Jolla, part numbers 200347 and 200420). Three RT-PCR fragments are amplified using an LA PCR Kit Ver. 2 using buffer containing 1.5 mM Mg+2 (TaKaRa Shuzo Co., Ltd., Japan, part number RR013A). Nested PCR is performed. In this reaction, a second PCR is performed using a pair of primers within the sequence amplified by the first PCR reaction. The cycling conditions for each amplification are: 10 min at 95°C, 35 cycles of 1 min at 60°C, 1 min at 72°C, and 1 min at 95°C, followed by 7 min at 72°C in a Perkin-Elmer 9600 PCR machine. The amplified fragments are purified using 96-well plate spin columns (Wang et al., *Anal. Biochem. 226*:85-90, 1995). DNA sequence is determined using an FS Dye-Terminator sequencing kit (Applied Biosystems Division of Perkin Elmer) and the specific primers described below. An automated Applied Biosystems ABI373A DNA Sequencer is used to determine the sequence. The amplified fragments and the appropriate primers are listed in Table 1, and the primer sequences are listed in Table 2.

The DNA sequences are aligned with the known sequence (Figure 2A) using the program Sequencher (Gene Codes, Michigan) to identify any discrepancies between patient samples and the reference sequence.

Table 1 PCR and sequence primers

Fragment	Primers Nested on cDNA		Coordinates	Sequence primers
	1st PCR	2nd PCR		
I	5EC, J	5EN, L	2947-5065	5EN, L, M, N, O, P, Q, R
II	5ED, P	5EE, B	1379-3391	5EE, 5EJ, 5EK, 5EL, 5EM, 5EB, 5EA, 5EN, B
III	5ES, 5EK	5ET, 5EH	75-1516	5ET, 5EX, 5E1, 5EP, 5EO, 5ED, 5EH

Table 2 Primer sequences

B	5'-CTTTATGAAGCCAATTTCTACCC	(SEQ ID No. 2)
J	5'-TTGCCTAGTGCAATTGGTCTCC	(SEQ ID No. 10)
L	5'-CCTATTTAATGGCACCCAAAATGC	(SEQ ID No. 12)
M	5'-CAGTCTATGGCCATCACATACTC	(SEQ ID No. 13)
N	5'-ACCGCTTGGGATAAGTGCATGC	(SEQ ID No. 14)
O	5'-GAGAAGAAGTCTAACTTGGAGAAG	(SEQ ID No. 15)
P	5'-TTCTGGTGACTGTACCATGATAC	(SEQ ID No. 16)
Q	5'-CCAAAGGAAGTGATACCAGCAAG	(SEQ ID No. 17)
R	5'-ACAGCAAGAAACATAATTGTTCTGG	(SEQ ID No. 18)
5EA	5'-GAACTTTGAAGTCCATCACGACC	(SEQ ID No. 19)
5EB	5'-GCATTAATAAAGCTGACATTCGCC	(SEQ ID No. 20)
5EC	5'-CATTACGGTGCTCCTAAGGACATG	(SEQ ID No. 21)
5ED	5'-GATGGATTTGAAGATGGAGTAGAAG	(SEQ ID No. 22)
5EE	5'-TGAAAGAGAATATGGAAAGAGCTTG	(SEQ ID No. 23)
5EH	5'-CATTGGGAGATAAATGCTCAGTAGA	(SEQ ID No. 80)
5EJ	5'-AGATGTACTTTGGGCATTCCAG	(SEQ ID No. 81)
5EK	5'-GCCATGACAGCAACATTATCTC	(SEQ ID No. 82)
5EL	5'-CTTACTGCTACTGCAAGTTCTTC	(SEQ ID No. 83)
5EM	5'-TCGATCAAAACCAGTACAGGTG	(SEQ ID No. 84)
5EN	5'-GCAGATGTAGGAGACAAATCATC	(SEQ ID No. 85)
5EO	5'-TCATCCAAAATCTCTAAATTTCGG	(SEQ ID No. 86)
5EP	5'-CTGAGGACCAGAAACTGTATGC	(SEQ ID No. 87)
5ES	5'-GCTGATTTGGTGTCTAGCCTGG	(SEQ ID No. 88)
5ET	5'-TGCCTGGGTTGCAGGCCTGC	(SEQ ID No. 89)
5EX	5'-TTGGAAACAACTGCACAGCAGC	(SEQ ID No. 90)

EXAMPLE 6

ISOLATION OF GENOMIC DNA CONTAINING WERNER'S SYNDROME GENE

To facilitate mutational analysis of the *WRN* gene, the intron-exon structure is determined. The *WRN* gene is located in the genomic sequence of P1 clone 2934. However, this clone only contains the 3' end of the gene (exons 21 to 35). Genomic clones containing the 5' end are obtained from a chromosome 8-specific cosmid library LA08NC01 (Wood et al. *Cytogenet. Cell Genet. 59*: 243, 1992) by screening for clones adjacent to P1 clone 2934. Briefly, this library is arrayed for PCR screening as described in Amemiya et al. (*Nucl. Acids Res. 20*: 2559, 1992). *WRN* containing cosmids are identified using primer sets 5E6/5EY, 5ED/5E12, and CD-A/CD-B (Table 3), which are derived from the *WRN* cDNA sequence (Figure 1; GenBank Accession No. L76937). Four walking steps yielded cosmids 193B5, 114D2, 78D8 and 194C3, which contained the remaining exons. Primers derived from the WRN cDNA were used for the initial sequence analysis of the cosmid clones. The resulting sequence (Figure 5) is compared to the cDNA sequence to identify intron-exon boundaries. Sequencing primers are then designed from the intron sequences to obtain sequence in the reverse direction and to obtain the second boundary defining the intron-exon junction. This strategy is used to define the exons not present in P1 clone 2934.

Table 3. Primer sequence and PCR conditions for *WRN* analysis

Region	Primer Sequence	Product Size (bp)	Mg^{+2} (mM)	pH
N-domain	5E6 5'-GATATTGTTTTGTATTTACCCATGAAGAC (SEQ ID No: 164 5EY 5'-TCCGCTGCTGTGCAGTTCTTTCC SEQ ID No: 165)	106	1.5	8.3

The annealing temperature was 60° C for all primer sets.

Table 4 presents a summary of the structure of the genomic WRN gene. The first column identifies the exon, the second column indicates the base numbers of the cDNA that are derived from the exon, the third column denotes the size of the exon in bp, the fourth column shows the sequence of the boundaries with intron sequences in lower case letters and exon sequences in upper case letters, the fifth column shows notable features of the exons.

Table 4. Intron-Exon Structure of the *WRN* Gene

Exon	cDNA Location	Exon Size (bp)	Intron-Exon Boundary Sequences	Exon Features
1	1-155	>155	....TTCTCGGGgtaaagtgtc (SEQ ID No. 169)	5'UTR
2	156-327	172	tacctctcagTTTTCTTT ...AAAGAAAGgtatgttgtt (SEQ ID No. 170)	5'UTR, ATG codon
3	328-440	113	taaactcaagGCATGTGT....GATATTAGgtaagtgatt (SEQ ID No. 171)	
4	441-586	146	ctcactttagCATGAGTC ...CATGTCAGgttggtatct (SEQ ID No. 172)	
5	587-735	149	aatgttacagTTTTTCCC ... ATAAAAAGgtaaaagcaa (SEQ ID No. 173)	
6	736-885	150	tcatttctagCTGAAATG... ATGCTTATgtacgtgctt (SEQ ID No. 174)	
7	886-955	70	tttttatagGCTGGTTT ... AAATAAAGgtatgttaag (SEQ ID No. 175)	
8	956-1070	115	ttccccctagAGGAAGAA....CCACGGAGgttaaatatt (SEQ ID No. 176)	
9	1071-1500	430	tttttttagGGTTTCTA....CTACTGAGgtactaaaat (SEQ ID No. 177)	
10	1501-[illegible]	81	tt[illegible]taaagCATTTAT[illegible] [illegible]TGCTTAAG[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]-1807	[illegible]	[illegible] (SEQ ID No. 180)	

Exon	cDNA Location	Exon Size (bp)	Intron-Exon Boundary Sequences	Exon Features
13	1808-1883	76	ttatttccagACTTTTTG . . TTTAAACCgtgagtataa (SEQ ID No. 181)	
14	1884-1951	68	caccttcaagAGTTCAGT . . . .GGCAACTGgtaagttgta (SEQ ID No. 182)	helicase motif I (5' end)
15	1952-2060	109	tcatttcaagGATATGGA . . . .CAGCTTAAgtaagtcatg (SEQ ID No. 183)	helicase motif I (3' end) arc la
16	2061-2129	69	cttcttatagAATGTCCA . . . ATTAAATTgtgagtaatt (SEQ ID No. 184)	
17	2130-2212	83	gtttttacagAGGTAAAT . . . TGATATTGgtaagtgata (SEQ ID No. 185)	
18	2213-2319	107	ttttttacagGTATCACG . . . TGCCAATGgtaagctttg (SEQ ID No. 186)	helicase motif II
19	2320-2504	185	catcattcagGTTCCAAT . . . .AAAACAAGgtaaggattt (SEQ ID No. 187)	helicase motif III
20	2505-2679	175	ttttctttagTTCCCACT . . . .AAATTCAGgtatgaggat (SEQ ID No. 188)	helicase motif IV
21	2680-2861	182	ttgttctcagTGTGTCAT . . . TTAAATAGgtaaaaaaaa (SEQ ID No. 189)	helicase motifs V and VI
22	2862-2963	102	taatcgacagGCACCTTC . . . .AGGAGACAgtatgtatta (SEQ ID No. 190)	
23	2964-3056	93	tcttgggtagAATCATCT . . . .AGGTCCAGgtaaagattt (SEQ ID No. 191)	
24	3057-3198	142	ttttatttagATTGGATC . . . .GAGGATCTgtaagtatat (SEQ ID No. 192)	
25	3199-3369	171	ctaatttcagAATTCTCA . . . CGAAAAAGgtaaacagtg (SEQ ID No. 193)	
26	3370-3464	95	ctttaatagGGTAGAAA . . . CTGCCTAGgtt.tatttt (SEQ ID No. 194)	
27	3465-3540	76	tattttttagTTCGAAAA . . . AGAAGAAGgtttgtttta (SEQ ID No. 195)	
28	3541-3614	74	ttaaatgcagTCTAACTT . . . AAAAAAAGgtacagagtt (SEQ ID No. 196)	
29	3615-3690	76	aatattttagTATCATGG . . . AGACTCAGgtaagagcttt (SEQ ID No. 197)	

Exon	cDNA Location	Exon Size (bp)	Intron-Exon Boundary Sequences	Exon Features
32	3919-4050	132	aattctgtagACAGACCT....TGCCTTTGgtaagtgtga (SEQ ID No. 200)	
33	4051-4213	163	ctttctctagAAGAGCAT....CAACTCAGgtgagaggca (SEQ ID No. 201)	
34	4214-4422	209	tcgtttacagATATGAGT....ATACTGAGgtattaatta (SEQ ID No. 202)	
35	4423-5190	768	tttcctacagACTTCATC.... (SEQ ID No. 203)	TAA codon, 3'UTR

Note. Exons are in uppercase and intron sequences are in lowercase letters.

As shown above, WRN contains a total of 35 exons ranging in size from 68 bp (exon 14) to 768 bp (exon 35). The coding region begins in the second exon (Table 2). As noted previously, there is a duplicated region in the WRN cDNA sequence which is 27 amino acids in length. This duplication is exactly conserved at the nucleotide level in cDNA. At the genomic level, the duplicated sequences were present as 2 exons (exons 10 and 11), each exon containing only the duplicated nucleotides. The intronic sequences adjacent to these 2 exons are also highly conserved, suggesting that the a relatively recent duplication event is responsible for these repeated exons. In addition, because the surrounding intronic sequences were conserved, it was not possible to design primers which could specifically amplify exons 10 and 11.

The helicase region of the WRN gene is contained in exons 14-21. Helicase motif 1 is split between exons 14 and 15 while the remaining motifs are each in an individual exon (Table 4). This region, from codon 569 to 859, has sequence similarity to the 7 signature helicase motifs. In addition, though the sequences between the motifs are not conserved, the spacing is very similar in genes from a wide range of species. For example, the helicase domains in the *E. coli RecQ* gene are found in a

EXAMPLE 7

IDENTIFICATION OF MUTATIONS

Initially, 4 different mutations in the C-terminal domain of *WRN* were identified. These mutations accounted for more than 80% of the Japanese WS patients examined. All 4 mutations are in the C-terminal domain region of *WRN* and the resulting predicted protein contained an intact helicase domain. Additional WS subjects are screened to identify further mutations. Genomic structure information is used to design PCR-primers for amplifying each exon, which is then subjected to DNA sequence analysis. Five additional *WRN* mutations are described; 2 are located in the consensus helicase motifs and another 2 are predicted to produce truncated proteins without the helicase domains. These mutations suggest that in at least some WS subjects, the enzymatic helicase activity is destroyed and support that complete loss-of-function of *WRN* gene product causes Werner's syndrome.

Although any cell may be used to isolate DNA, PBMC are preferred. As above, PBMC are obtained by venipuncture and subsequent hypotonic lysis of erythrocytes. PBMC are lysed by the addition of detergent, such as 0.5% NP-40, 0.5% Triton-X100, or 0.5% SDS. If a non-ionic detergent is used, no further purification of DNA is necessary, but proteinase K treatment, and subsequent heat killing of the enzyme (95°C for 10 minutes) is required. Genomic DNA is amplified according to the PCR conditions recited above using the primers listed in Table 5. Exons 9 and 10 are contained in a region of DNA that is duplicated. The primer pair for exon 9 and 10 anneals to sequences outside the duplication. Amplified product is analyzed by DNA sequence determination, hybridization with allele-specific probe, or other mutation detection method. When DNA sequences are determined, the sequence of the amplified exon is aligned with the known sequence (Figure 2A) and any discrepancies between patient samples and the reference sequence are identified.

Table 5

PCR Fragment	Primer Sequence	Product Size (bp)	Mg^{++} (mM)	pH
exon 1	A 5'-AGGGCCTCCACGCATGACGC (SEQ ID No. 92) B 5'-AGTCTGTTTTTCCAGAATCTCCC (SEQ ID No. 93)	583	1.5	8.3
exon 2	A 5'-CCTATGCTTGGACCTAGGTGTC (SEQ ID No. 94) B 5'-GAAGTTTACAAGTAACAACTGACTC (SEQ ID No. 95)	339	1.5	8.3
exon 3	A 5'-ACTATAAATTGAATGCTTCAGTGAAC (SEQ ID No. 96) B 5'-GAACACACCTCACCTGTAAAACTC (SEQ ID No. 97)	316	1.5	8.3
exon 4	E 5'-GGTAAACCACCATACCTGGCC (SEQ ID No. 98) F 5'-GTACATATCCTGGTCATTTAGCC (SEQ ID No. 99)	691	1.5	8.3
exon 5	B 5'-ATTCAGATAGAAAGTACATTCTGTG (SEQ ID No. 100) E 5'-GTTAAGAAATACTCAAGGTCAATGTG (SEQ ID No. 101)	369	1.5	8.3
exon 6	A 5'-GGTTGTATTTTGGTATAACATTTCC (SEQ ID No. 102) B 5'-ATATTTTGGTAGAGTTTCTGCCAC (SEQ ID No. 103)	374	1.5	8.3
exon 7	A 5'-CTCTTCGATTTTTCTGAAGATGGG (SEQ ID No. 104) B 5'-CCCTAATAGTCAGGAGTGTTCAG (SEQ ID No. 105)	291	1.5	8.3
exon 8	A 5'-GGAAAGAAAATGAAAATTTGATCCC (SEQ ID No. 106) B 5'-CAGCCTTAATGAATAGTATTCTTCAC (SEQ ID No. 107)	316	4.0	8.3
exon 9	C 5'-ATTGATCTTTTAAGTGAAGGTCAGC	668	1.5	8.3

E 5'-GAAATTATCTAAAAATGGATC

PCR Fragment	Primer Sequence	Product Size (bp)	Mg^{+2} (mM)	pH
	(SEQ ID No. 111)			
exon 13	A 5'-TAACCCATGGTAGCTGTCACTG (SEQ ID No. 112) B 5'-CTGTTGCTGTTAAGCAGACAGG (SEQ ID No. 113)	285	1.5	8.3
exon 14	C 5'-TTGAATGGGACATTGGTCAAATGG (SEQ ID No. 114) D 5'-GTAGTTGCATTTGTATTTTGAGAGT (SEQ ID No. 115)	348	1.5	8.3
exon 15	C 5'-GTAAAAAGAAATGAAAGCATCAAAGG (SEQ ID No. 116) D 5'-TCACCCACAGAAGAAAAAAAGAGG (SEQ ID No. 117)	246	4.0	8.3
exon 16	A 5' CAAAAAAGAAAATTGCAAAGAACAGG (SEQ ID No. 118) B 5'-CAGCAACATGTAATTCACCCACG (SEQ ID No. 119)	282	4.0	8.3
exon 17	5' GAAGAGACTGGAATTGGGTTTGG (SEQ ID No. 120) 5'-ATAGAGTATCATGGGATAAGATAGG (SEQ ID No. 121)	532	1.5	8.3
exon 18	A 5'-TTCTCCTTTGGAGATGTAGATGAG (SEQ ID No. 122) B 5'-TCTTCAGCTTCTTTACCACTCCCCA (SEQ ID No. 123)	273	4.0	10
exon 19	A 5'-CATGGTGTTTGACAACAGGATGG (SEQ ID No. 124) B 5'-GTTAAATATGCATTAGAAGGAAATCC (SEQ ID No. 125)	396	4.0	9.0
exon 20	A 5'-ATAAAACCAAACGGGTCTGAAGC (SEQ ID No. 126) B 5'-AAAAGAAGTATTCAATAAAGATCTGG (SEQ ID No. 127)	342	4.0	8.3
exon 21	A 5'-AATTCCACTTTGTGCCAGGGACT (SEQ ID No. 128) B 5'-ACTTGGGATACTCCAAATAGCCT (SEQ ID No. 129)	397	1.5	9.0

PCR Fragment	Primer Sequence	Product Size (bp)	Mg^{++} (mM)	pH
exon 23	A 5'-CTGAAGTCAAATAATGAAGTCCCA (SEQ ID No. 132) B 5'-GTTTGCTTTCTCATATCTAAACACA (SEQ ID No. 133)	360	4.0	8.3
exon 24	A 5'-CTTGTGAGAGGCCTATAAACTGG (SEQ ID No. 134) B 5'-GGTAAACAGTGTAGGAGTCTGC (SEQ ID No. 135)	267	1.5	8.3
exon 25	C 5'-GCTTGAAGGATGAGGCTCTGAG (SEQ ID No. 136) D 5'-TGTTCAGAATGAGCACGATGGG (SEQ ID No. 137)	461	1.5	8.3
exon 26	A 5'-CTTGTGAGAGGCCTATAAACTGG (SEQ ID No. 138) B 5'-GGTAAACAGTGTAGGAGTCTGC (SEQ ID No. 139)	267	1.5	8.3
exon 27	A 5'-GCCATTTTCTCTTTAATTGGAAAGG (SEQ ID No. 140) B 5'-ATCTTATTCATCTTTCTGAGAATGG (SEQ ID No. 141)	274	1.5	8.3
exon 28	A 5'-TGAAATAGCCCAACATCTGACAG (SEQ ID No. 142) B 5'-GATTAATTTGACAGCTTGATTAGGC (SEQ ID No. 143)	291	1.5	8.3
exon 29	A 5'-TGAAATATAAACTCAGACTCTTAGC (SEQ ID No. 144) B 5'-GTACTGATTTGGAAAGACATTCTC (SEQ ID No. 145)	303	1.5	8.3
exon 30	A 5'-GATGTGACAGTGGAAGCTATGC (SEQ ID No. 146) B 5'-GGAAAAAATGTGGTATCTGAAGCTC (SEQ ID No. 147)	307	1.5	8.3
exon 31	A 5'-AAGTGAGCAAATGTTGCTTCTGG (SEQ ID No. 148) B 5'-TCATTAGGAAGCTGAACATCAGC (SEQ ID No. 149)	304	1.5	8.3

PCR Fragment	Primer Sequence	Product Size (bp)	Mg^{++} (mM)	pH
exon 33	A 5'-TAAAGGATTAATGCTGTTAACAGTG (SEQ ID No. 152) B 5'-TCACACTGAGCATTTACTAGCTG (SEQ ID No. 153)	360	1.5	8.3
exon 34	C 5' GCAAAGGAAATGTAGCACATAGAG (SEQ ID No. 154) D 5'-AGGCTATAGGCATTTGAAAGAGG (SEQ ID No. 155)	491	1.5	8.3
exon 35	A 5'-GTAGGCTCCCAGAAGACCCAG (SEQ ID No. 156) B 5'-GAAAGGATGGGTGTGTATTCAGG (SEQ ID No. 157)	406	1.5	8.3
mutation 7	GD A 5'-ACAGGCCATAGTTTGCCAACCC (SEQ ID No. 158) GC D 5'-TGGTATTAGAATTTCCCTTTCTTCC (SEQ ID No. 159)	426	1.5	9.0
DJG RT-PCR	5EE 5'-TGAAAGAGAATATGGAAAGAGGCTTG (SEQ ID No. 160) B 5'-CTTTATGAAGCCAAATTTCTACCC (SEQ ID No. 161)	2002	1.5	8.3
P2934AT1	A 5'-TCAAAATCAGTCGCCTCATCCC (SEQ ID No. 162) B 5'-CAATGTATCAGTCAGGGTTCACC (SEQ ID No. 163)	168	2.0	8.3

The annealing temperature was 60° C for all primer sets.

Mutations are detected by amplifying *WRN* exons from genomic DNA and directly cycle-sequencing the PCR products by dye-terminator cycle sequencing (Perkin Elmer) and an ABI373 automated DNA sequencer. Prior to sequencing, the PCR-amplified exon fragments were purified using a QIAquick 8 PCR purification kit (Qiagen). The resulting sequences are aligned by FASTA analysis (GCG). Nucleotide differences between WS and controls are subsequently confirmed by sequencing the reverse strand

consequences of splice-junction mutations. RT-PCR products were synthesized from

mRNA isolated from lymphoblastoid cell lines (Qiagen Oligotex, Qiagen). The large genomic deletion was detected in genomic DNA using long-range PCR (Expand Long Template PCR System, Boehringer Mannheim).

Diagnostic Criteria. WS patients were from an International Registry of Werner's Syndrome subjects. Diagnostic criteria are based on the following signs and symptoms (Nakura et al. 1994). Cardinal signs are: 1) bilateral cataracts; 2) characteristic dermatological pathology (tight skin, atrophic skin, pigmentary alterations, ulceration, hyperkeratosis, regional subcutaneous atrophy) and characteristic facies ("bird" facies); 3) short stature; 4) paternal consanguinity (3rd cousin or greater) or affected sibling; 5) premature greying and/or thinning of scalp hair; 6) positive 24-hour urinary hyaluronic acid test, when available). Further criteria are: 1) diabetes mellitus; 2) hypogonadism (secondary sexual underdevelopment, diminished fertility, testicular or ovarian atrophy); 3) osteoporosis; 4) osteosclerosis of distal phalanges of fingers and/or toes (X-ray diagnosis); 5) soft tissue calcification; 6) evidence of premature atherosclerosis (e.g. history of myocardial infarction); 7) mesenchymal neoplasms, rare neoplasms or multiple neoplasms; 8) voice changes (high pitched, squeaky or hoarse voice); 9) flat feet. Diagnostic classifications are as follows: "Definite", all cardinal signs (#6 when available) and any 2 others; "Probable", the first 3 cardinal signs and any 2 others; "Possible", either cataracts or dermatological alterations and any 4 others; "Excluded", onset of signs and symptoms before adolescence (except short stature since current data on pre-adolescent growth patterns is inadequate) or a negative hyaluronic acid test. Family designations are as previously used (Nakura et al. 1994; Goddard et al. 1996; Yu et al. 1996).

Mutations in WS Subjects. Initial screening of the WRN gene was based on sequence from only the 3' end of the gene (exons 23-35). Thus the first 4 mutations (designated 1-4, Table 3) were in the region 3' to the helicase domains. In this mutation screening, primers amplify exons 2-35 along with approximately 80 bp of

screened for mutations. These subjects were selected based on haplotype analysis that

suggested that each subject might have a different mutation (Yu et al. 1994; Goddard et al. 1996). A total of 30 Japanese and 36 Caucasian subjects were ultimately screened for each mutation by DNA sequence analysis of the appropriate exon.

Table 6. Summary of WRN Mutations

Mutation	Codon	Exon	Type of Mutation	Nucleotide Sequence	Comment	Predicted Protein Length
none						1432
1	1165	30	substitution	CAG (Gln) to TAG (terminator)	nonsense	1164
2	1305	33	substitution	CGA (Arg) to TGA (terminator)	nonsense	1034
3	1230	32	4 bp deletion	gtag-ACAG to gt-AG	4 bp deletion at splice-donor site	1247
4	1047-1078	24	substitution	tag-GGT to tac-GGT	substitution at splice-donor site	1060
5	369	9	substitution	CGA (Arg) to TGA (terminator)	nonsense	368
6	889	22	substitution	CGA (Arg) to TGA (terminator)	nonsense	888
7	759-816	20	substitution	CAG-gta to CAG-tta	substitution at splice-receptor site	760
8	389	9	1 bp deletion	AGAG (Arg) to GAG (Glu)	frame-shift	391
9	697-942	19-23	deletion (~15 kb)	-	genomic deletion	1186

Table 7.
Mutation Status of WS Subjects[1]

Mutation	Japanese WS Subjects		Non-Japanese WS Subjects	
	Homozygous	Heterozygous	Homozygous	Heterozygous
1	SY[D]			
2	HH[D], HM[D], MH[M], NN[D]		GAR[D]	
3			SYR[I]	
4	FJ[D], FUW[D], HA[I], HW[D], IU[D], JO1[D], JO2[D], KAKU[P], KY[D], MCI[D], MIE2[I], SK[D], ST[D], TH[I], TK[M], TO[D], ZM[D], 78-85[I]			
5	KO[D], OW[P]	KUN[I]	EKL[D], AG0780[I], AG4103[M]	DJG[P], CP3[I], NF[M]
6			CTA[D]	SUG1[P]
7	WKH[D]			
8				FES[I]
9				DJG[P], SUG1[P]

[1]The diagnostic classification is as previously described (Nakura et al. 1994). Diagnosis categories: [D] Definite; [P] Probable; [M] Possible; [I] Insufficient data. The country of origin (ethnic group) of non-Japanese subjects are: AG00780, USA (Caucasian); AG04103, USA (Caucasian); CTA, England (India, East African, Asian); CP3, France (Caucasian); DJG, Germany (German); EKL, Switzerland (German); FES, Germany (German); NF, France (Caucasian); SUG, USA (Caucasian); SYR, Syria (Syrian). AG04103 and AG00780 were obtained as cell lines from the Aging Cell Repository (Camden, New Jersey).

Five new WS mutations were detected in the WRN gene (designated 5-9, Table 6). Two of the mutations (5 and 6) were single base substitutions creating nonsense codons. Mutation 5 results in a C→T transition changing an Arg to a

Caucasian subjects were homozygous, and 1 Japanese and 3 Caucasians were heterozygous for this mutation (Table 7). Mutation 6 is also a C→T transition changing

an Arg to a nonsense codon. One Caucasian WS subject was homozygous for this mutation, and a second was a compound heterozygote. The predicted protein product is 888 amino acids. A third substitution mutation (mutation 7) was a G→T change at a splice-receptor site, generating a truncated mRNA devoid of exon 20 and a prematurely terminated WRN protein at amino acid 760. A single Japanese WS subject was homozygous for this mutation.

Two deletions were observed. One (mutation 8) is a 1 bp deletion at codon 389 resulting in a frame shift and a predicted truncated protein 391 amino acids long. This mutation is found in one Caucasian patient as a heterozygote. The second (mutation 9) is a much larger deletion. This deletion was first observed in RT-PCR experiments when 2 different RT-PCR products were obtained from RNA prepared from subject DJG. RT-PCR products produced by primers 5EE and B (Table 5) yielded 2 different products, one with the expected size of 2009 bp, and a second, shorter product approximately 700 bp smaller. The DNA sequence of the shorter product revealed that exons 19 through 23 were missing. To further establish the nature of this mutation, primers (exon 18A and exon 24A, Table 5) derived from the exons flanking this potential gross deletion (exons 18 and 24) were used to amplify genomic DNA from subject DJG using a long-range PCR protocol. A single 5 kb fragment was observed corresponding to the shorter RT-PCR product. (The normal fragment, which is estimated to be > 20 kb was not observed.) The complete DNA sequence of this 5 kb fragment was determined and contained the expected 3' and 5' ends of exons 18 and 24, respectively. The exonic sequences were separated by intronic sequences adjacent to the 3' and 5' end of exons 18 and 24, respectively. No sequences from exons 19-23 were found in the 5 kb fragment. In other subjects and controls, the intronic sequence in the intron 3' to exon 18 contained 531 bp of unique sequence followed by a 241 bp Alu repeat element. Likewise, for the region 5' to exon 24, there is an Alu repeat element separated from exon 24 by 3,460 bp of unique sequence. The 4938 bp

recombination error at 2 highly homologous Alu elements within the WRN gene. A

primer set, GD-A and GD-D (Table 5) was designed to specifically amplify a short fragment (426 bp) across this junction point. A single additional Caucasian WS patient, SUG, was shown to contain this genomic deletion. Further PCR amplification of the exons within this deleted region demonstrated that both DJG and SUG are heterozygous for this mutation.

Origins of WRN Mutations. Because multiple subjects have the same mutation and because the same mutation was observed in different ethnic groups, at least some of the mutations likely originated in common founders. Evidence for a common founder was examined using 2 short tandem repeat polymorphisms (STRPs) within the WRN gene. These STRPs, D8S2162 and p2934AT1, were isolated from the same P1 clone (p2934) and are within 17.5 kb of each other. While D8S2162 is not particularly polymorphic (heterozygosity = 54% in Japanese and 70% in Caucasians) and is primarily a 2 allele system (140 and 142 bp alleles), p2934AT1 is highly polymorphic (heterozygosity = 78% in both Japanese and Caucasian populations). For mutation 4, which has only been observed in Japanese subjects, all but 1 subject had the D8S2164/p2934AT1 haplotype of 140-148 (Table 8). The single exception, JO2, has the haplotype 140-150, with the p2934AT1 allele being 2 bp different from the 148 bp allele observed in other subjects with mutation 4. This 2 bp difference may be the result of a 2bp mutation, as is commonly observed in dinucleotide repeat STRP loci (Weber and Wong, 1993). The haplotype data is consistent with a common Japanese founder and is consistent with the linkage disequilibrium observed in the same Japanese subjects for other markers in the WRN region (Yu et al. 1994; Goddard et al., 1996). For mutations 2 and 5, in the Japanese, the 896R18-p2934AT1 haplotypes for the small number of available subjects, are consistent with common founders for each mutation. However, the non-Japanese subjects with mutations 2 and 5 have discordant p2934AT1 genotypes when compared to Japanese subjects with the same mutations. These results do not support a common founder for both Japanese and non-Japanese subjects with

discordant for p2934AT1 (e.g. compare AG00780 to FKI). It should be noted that

absence of evidence for a common founder does not necessarily exclude the possibility of a single originating mutational event. Intragenic recombination and/or mutations creating new alleles at the 2 STRP loci could, over time, obscure the origins of the different WRN mutations.

Table 8. STRP Genotypes at the WRN gene[1].

Subject	Ethnic Group	Mutation	y896r18	p2934at1
FJ, FUW, HA, HW, JO1, KAKU, KY, MIE2, TO	Japanese	4	140/140	148/148
JO2	Japanese	4	140/140	150/150
HM, MH, NN,	Japanese	2	140/140	144/144
GAR	Hispanic	2	140/140	156/156
OW, KO	Japanese	5	140/140	148/148
AG00780	Caucasian	5	142/142	136/136
EKL, AG04103	Caucasian	5	142/142	128/128
CP3	Caucasian	5/?	142/150	128/142
KUN	Japanese	5/?	140/142	128/148
DJG	Caucasian	5/9	140/142	128/del[2]

[1]Genotype data for HH, SK, ST, TH, TK, and ZM was not available For y896R18, alleles in bp (frequency for Caucasians, frequency for Japanese) were as follows: 136 (0.030, 0.025); 138 (0.020, 0.010); 140 (0.460, 0.576); 142 (0.337, 0.359); 144 (0.084, 0.010); 146 (0, 0.010); 148 (0.009, 0.010); 150 (0.059, 0). For p2934AT1, alleles in bp (Caucasian frequency, Japanese frequency) were as follows: 114 (0.006, 0); 122 (0, 0.009); 124 (0.011, 0); 128 (0.253, 0.079); 130 (0, 0.018); 132 (0.006, 0.009); 134 (0.046, 0.096); 136 (0.086, 0.009); 138 (0.011, 0); 140 (0.034, 0); 142 (0.052, 0.035); 144 (0.023, 0.061); 146 (0.023, 0.053); 148 (0.034, 0.132); 150 (0.034, 0.105); 152 (0.057, 0.123); 154 (0.063, 0.088); 156 (0.086, 0.070); 158 (0.098, 0.070); 160 (0.046, 0.018); 162 (0.029, 0.009); 166 (0, 0.009); 168 (0, 0.009).

The 5 mutations identified here demonstrate that WS mutations are not restricted to the 3' end of the gene, but are also found in other regions of WRN. In addition, mutations 5 and 7-9 each disrupt either part or all of the helicase region. Thus the WS subjects homozygous for this mutation will completely lack the WRN helicase

probably results in complete loss of all activity of the WRN protein. However, the WS phenotype in these subjects is not appreciably distinct from the WS phenotype

generated by the other mutations described here. Thus, all mutations in the WS gene may be complete loss of function mutations.

EXAMPLE 8

IDENTIFICATION OF MOUSE WRN GENE

The mouse WRN cDNA was isolated by screening a mouse splenocyte cDNA library at low strengency with human WRN cDNA as probe. The mouse cDNA sequence is presented in Figure 9. The homology between human and mouse WRN cDNA sequence is about 80%. On the amino acid level, the human and mouse WRN gene product show about 90% identity. Notably, the repeated exon in human WRN cDNA (exons 10 and 11) is only present once in mouse WRN cDNA.

Genomic mouse WRN clone was isolated by using mouse WRN specific primers to screen mouse genomic BAC library. The genomic DNA sequence is presented in Figure 6.

The genomic DNA sequence is presentd in Figure 7 and SEQ ID NOS: 207-209. The DNA sequence is presented in Figure 6 and SEQ ID NOS: 205 and 206.

EXAMPLE 9

LOCALIZATION OF THE WRN GENE PRODUCT

A rabbit polyclonal antiserum raised to a peptide of WRN gene product is used in an indirect immunofluorescence assay to determine the intracellular localization of the WRN protein.

A rabbit polyclonal antiserum is raised to the peptide Phe-Pro-Gly-Ser-Glu-Glu-Ile-Cys-Ser-Ser-Ser-Lys-Arg (FPGSEEICSSSKR) (SEQ ID NO: 204) by standard methods (*see* Harlow and Lane, *Antibodies, A Laboratory Manual*, CSH Press, Cold Spring Harbor, 1989; *Current Protocols in Immunology*, Greene Publishing, 1995). The peptide corresponds to residues 1375 through 1387 of the WRN

fixed with 3% paraformaldehyde and permeabilized for 2 min with a buffer containing

0.5% Triton X-100, 10 mM PIPES, pH 6.8, 50 mM NaCl, 300 mM sucrose, and 3 mM $MgCl_2$ (*see for example*, Fey et al., *J. Biol. Chem. 98*: 1973, 1984). The cells are then stained for 20 min with a suitable dilution of the anti-peptide antibody (1:1500), washed, stained with a suitable second antibody (*e.g.*, FITC-conjugated goat anti-rabbit antibody), washed, and mounted for visualization by gluorescence microscopy. Control stains include bis-benzimidine (Sigma, St. Louis, MO), which stains DNA, and phalloidin (Molecular Probes, OR, BODIPY 558/568 phalloidin), which stains filamentous actin.

As seen in Figure 9, the WRN gene product is almost entirely located in the nucleus. Nuclear staining is readily noted in the epithelial cells at the bottom left in panel A. These cells are close to the periphery of the expanding clone of human prostate epithelial cells. Cells that are not rapidly dividing (*e.g.*, cells closer to the center of the clone), such as those seen in the upper right of panel A, are stained in both the cytoplasm and nucleus. The location and size of the nuclei in these cells is shown by staining DNA with the intercalating dye bis-benzimidine (Hoeschst 33258), panel B. The overall size of the cells and in some cases key cytoskeletal features are revealed by staining for F-actin as shown in panel C.

EXAMPLE 10

ISOLATION OF A PROTEIN THAT BINDS TO THE WRN GENE PRODUCT

A yeast 2-hybrid interaction screen (Hollenberg et al., *Mol. Cell Biol. 13*: 3813, 1995) is used to identify and isolate a cellular protein that binds to the carboxy-terminal 443 amino acids (residues 990 through 1432) of the WRN gene product.

A library of 1.1 x 106 independent cDNA clones generated from RNA isolated from stimulated human peripheral blood mononuclear cells is generated in pACT-2 (Clontech, Palo Alto, CA) that creates cDNA/GAL4 activation domain fusions is co-transfected into yeast containing pLEXA with the WRN gene fragment to generate

for histidine. [illegible] colonies grew on this medium. Of these, 60 were cured of the pLEXA

plasmid by growth on medium containing cycloheximide and mated with a yeast strain expressing a fusion of a "sticky" laminin and the GAL4 activation domain. 19 clones did not activate the sticky protein and underwent DNA sequence analysis. Of these, 6 contained sequences that did not match any sequence in GenBank by BLAST search. Two other clones encoded carnitine palmitoyl transferase I and prolyl 4-hydroxylase B subunit. Six independent clones encoded a 70K component of the U1 snRNP complex (GenBank Accession No. M22636). Moreover, all six derived from the RNA recognition motif region of the 70K protein.

From the foregoing, it will be appreciated that, although specific embodiments of this invention have been described herein for the pruposes of illustration, various modifications may be made without departing from the spirit and scope of the invention. Accordingly, the invention is not limited except by the appended claims.

Claims

We claim:

1. An isolated nucleic acid molecule encoding a WRN gene product.

2. An isolated nucleic acid molecule selected from the group consisting of:

(a) an isolated nucleic acid molecule as set forth in the Figures or complementary sequence thereof;

(b) an isolated nucleic acid molecule that specifically hybridizes to the nucleic acid molecule of (a) under conditions of high stringency; and

(c) an isolated nucleic acid that encodes a WRN gene product.

3. An expression vector, comprising a promoter operably linked to a nucleic acid molecule according to any one of claims 1 or 2.

4. The expression vector according to claim 3 wherein said promoter is selected from the group consisting of CMV I-E promoter, SV40 early promoter and MuLV LTR.

5. The expression vector according to claim 3 wherein said promoter is a tissue-specific promoter.

6. A viral vector capable of directing the expression of a nucleic acid molecule according to claims 1 or 2.

7. The viral vector according to claim 6 wherein said vector is selected

8. A host cell carrying a vector according to any one of claims 3 to 7.

9. The host cell according to claim 8 wherein said cell is selected from the group consisting of human cell, dog cell, monkey cell, rat cell and mouse cell.

10. An isolated protein comprising a WRN gene product.

11. An antibody which specifically binds to the protein according to claim 10.

12. The antibody according to claim 11 wherein said antibody is a monoclonal antibody.

13. The antibody according to claim 11 wherein said antibody is selected from the group consisting of an Fab fragment, an Fv fragment and a single chain antibody.

14. A hybridoma capable of producing an antibody according to claim 12.

15. A nucleic acid probe which is capable of specifically hybridizing to a WRN gene under conditions of high stringency.

16. A pair of primers capable of specifically amplifying all or a portion of a nucleic acid molecule according to any one claims 1 or 2.

17. A transgenic animal whose germ cells and somatic cells contain a WRN gene which is operably linked to a promoter effective for the expression of said gene, said gene being introduced into said mouse, or an ancestor of said mouse, at an embryonic

18. The transgenic animal according to claim 17 wherein the animal is selected from the group consisting of a mouse, a rat and a dog.

19. The transgenic animal according to claim 17 wherein WRN is expressed from a vector according to any one of claims 3 to 7.

20. An agonist of a WRN gene product.

21. An antagonist of a WRN gene product.

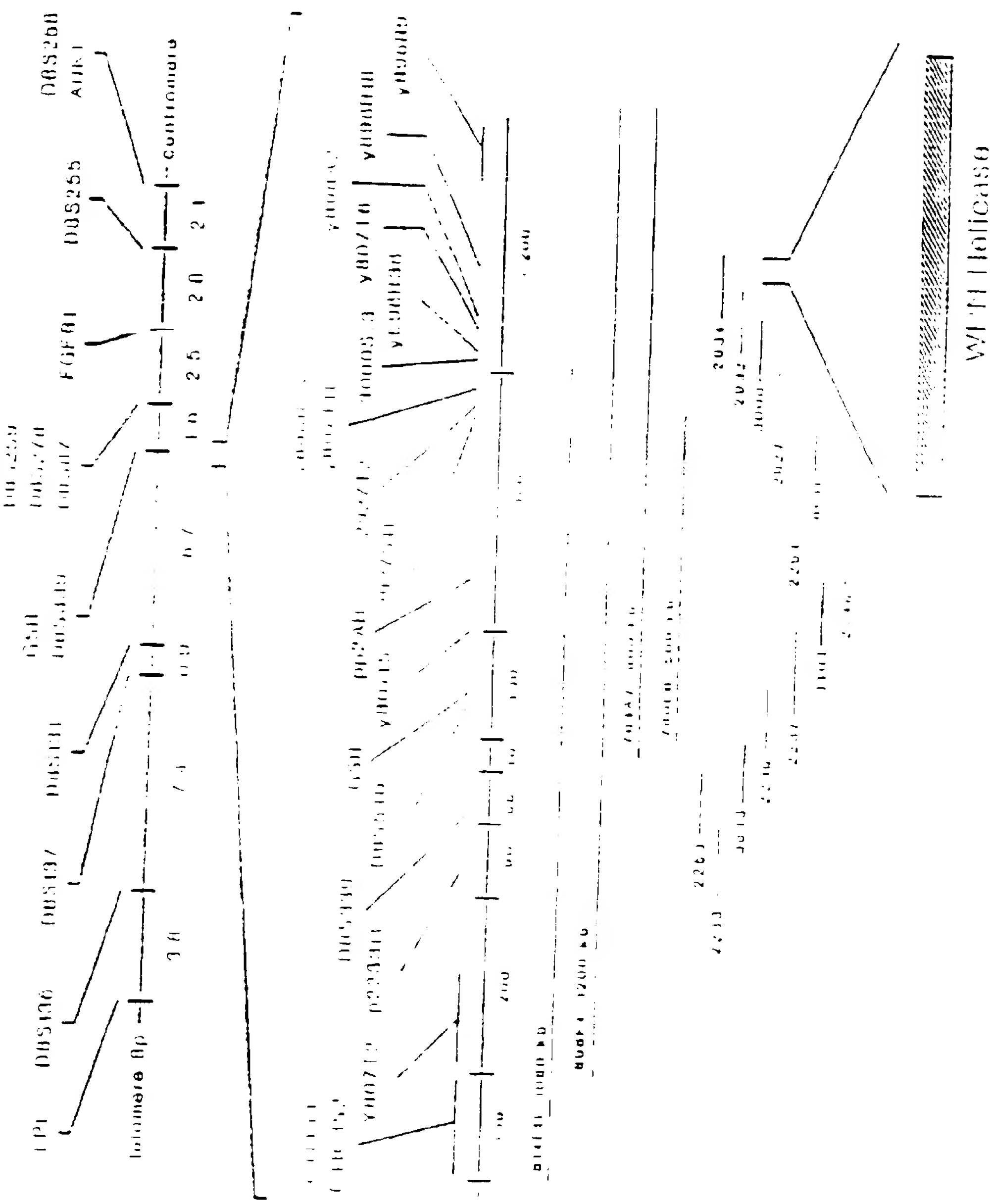

Telomere 8p
Centromere
D8S136
D8S137
D8S131
FGFR1
D8S255
D8S258
WRN Helicase

FIGURE 1

FIGURE 2A

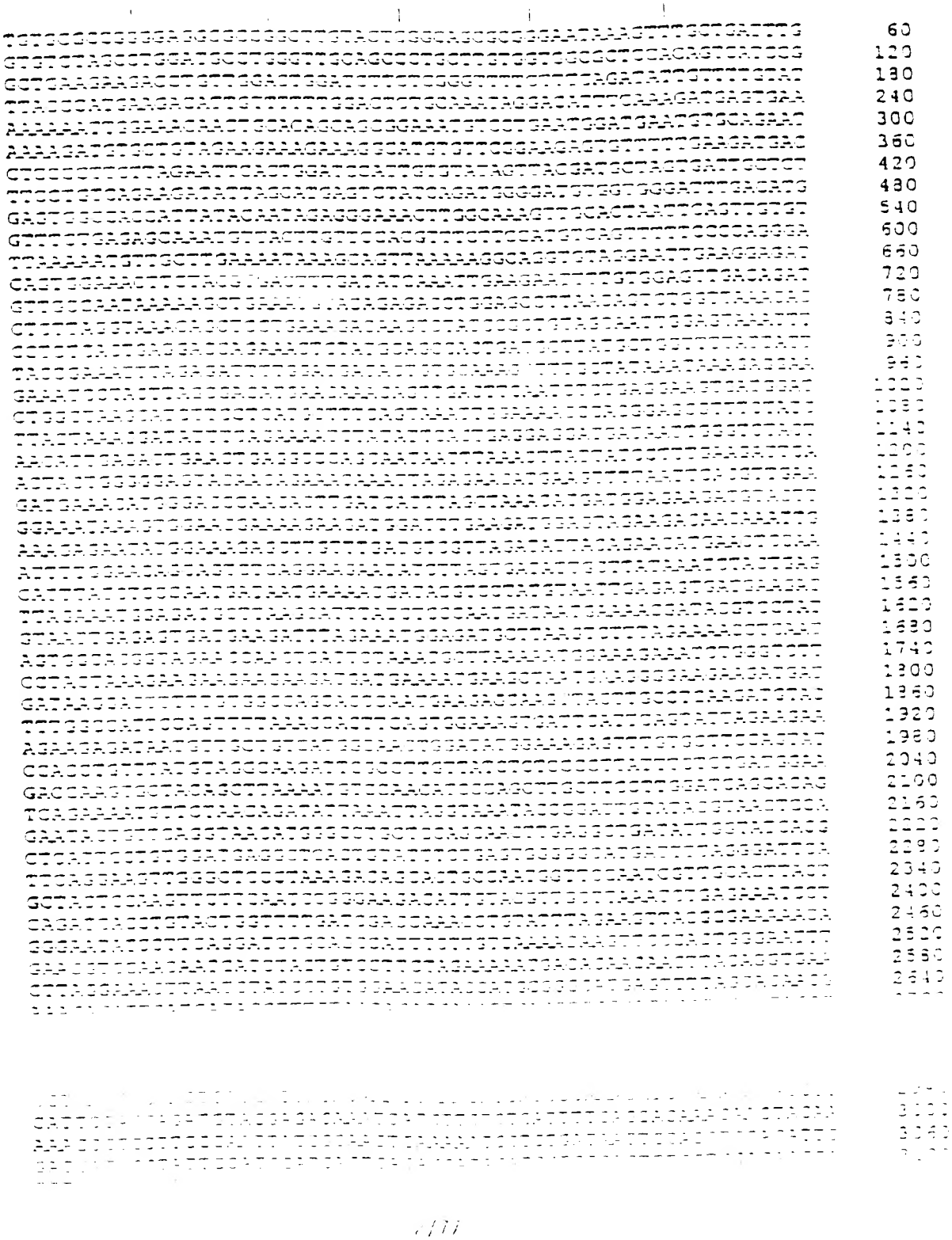


FIGURE 2A (CONT.)

FIGURE 2B

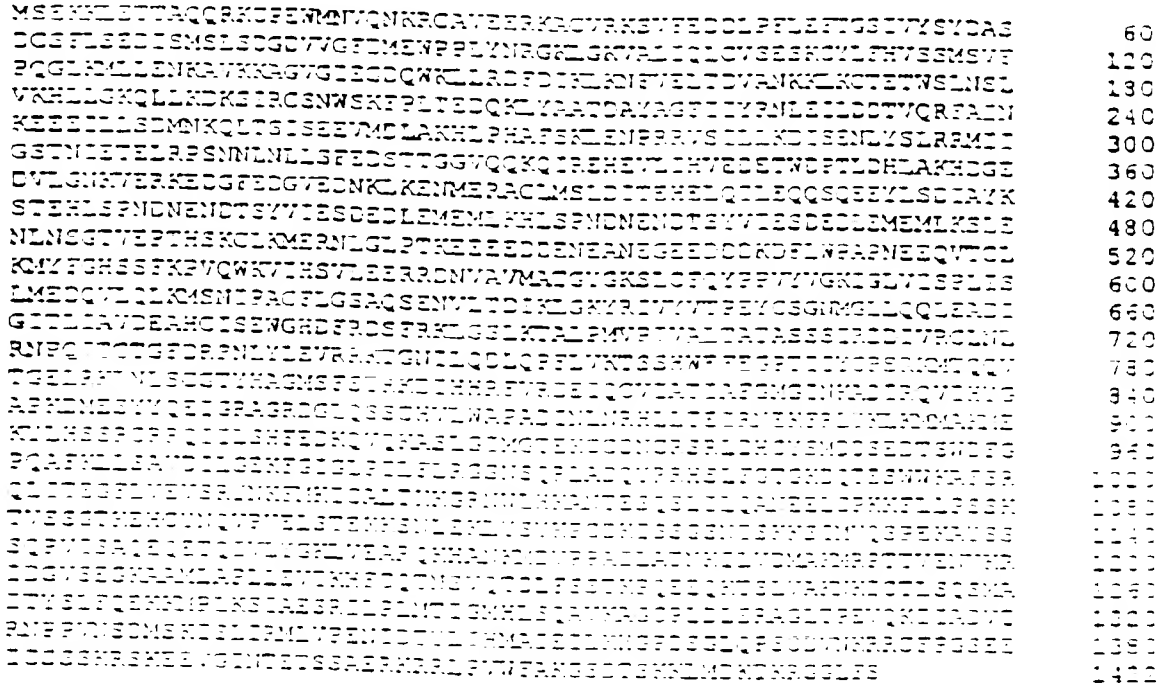


FIGURE 3A

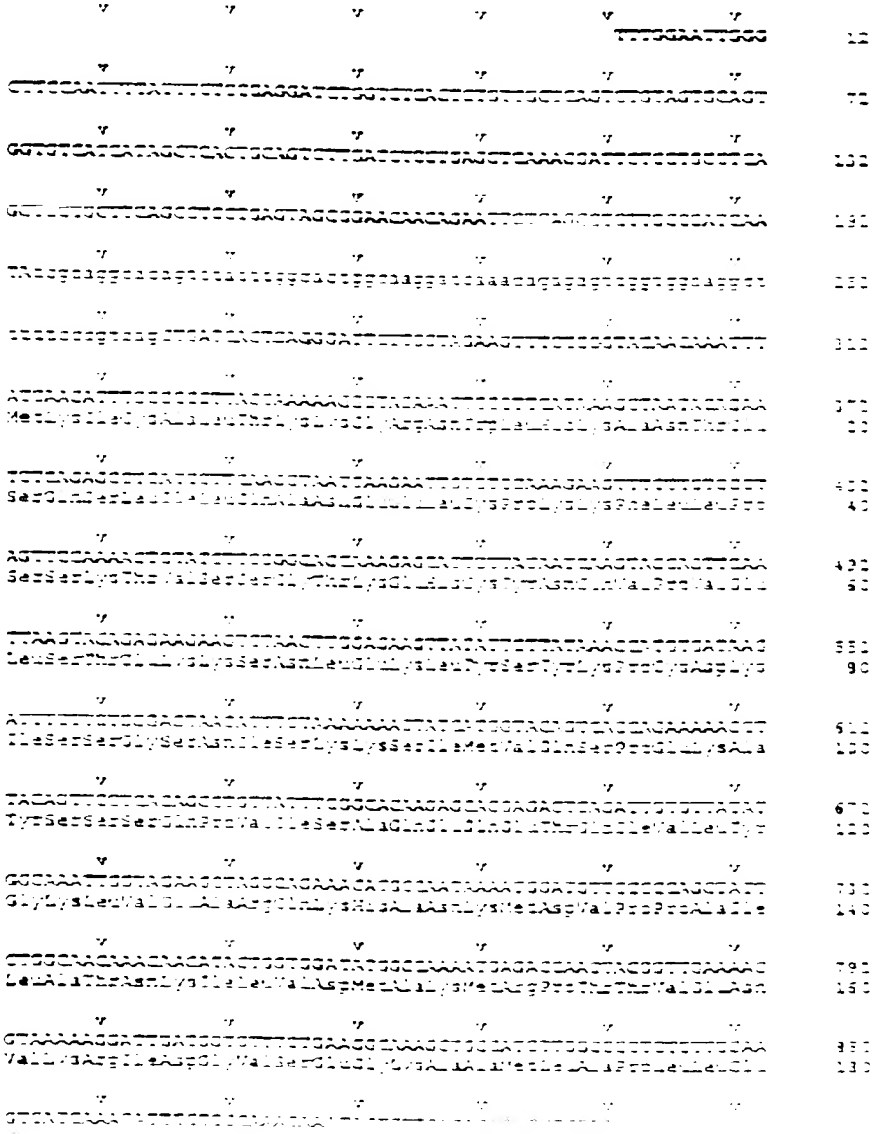


FIGURE 3B

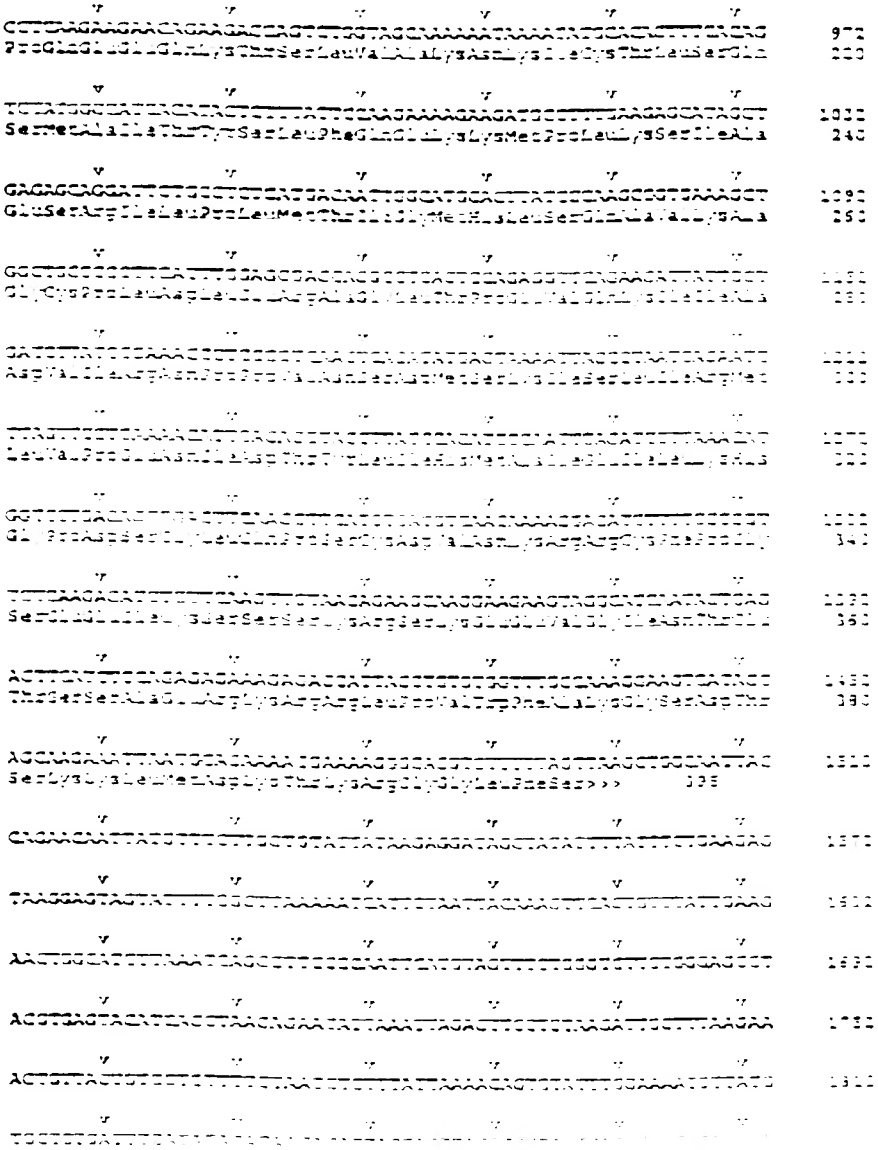


FIGURE 3C

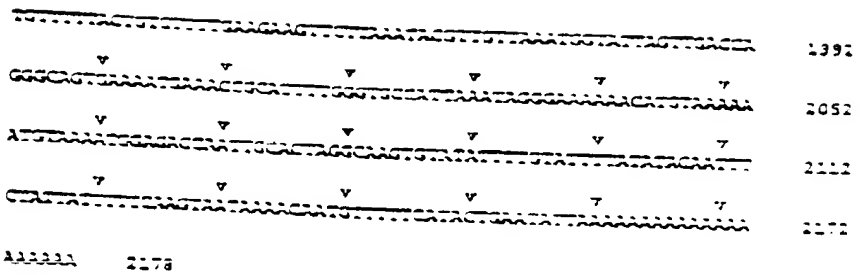


FIGURE 4A

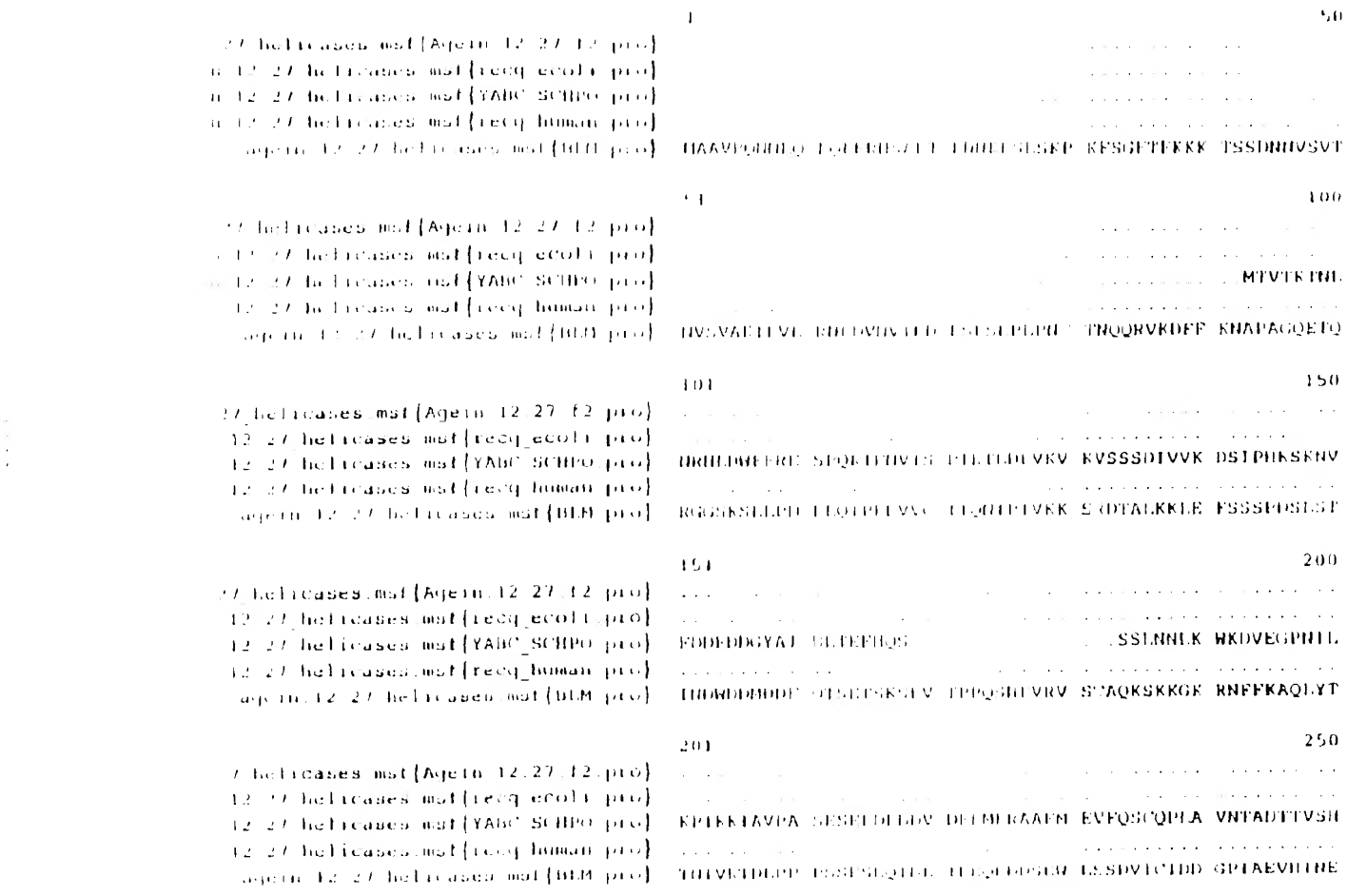

27_helicases.msf{Ageln_12_27_f2_pro}
12_27_helicases.msf{recq_ecoli_pro}
12_27_helicases.msf{YABC_SCHPO_pro}
12_27_helicases.msf{recq_human_pro}
Ageln_12_27_helicases.msf{BLM_pro}
1
50
100
150
200
250

FIGURE 4B

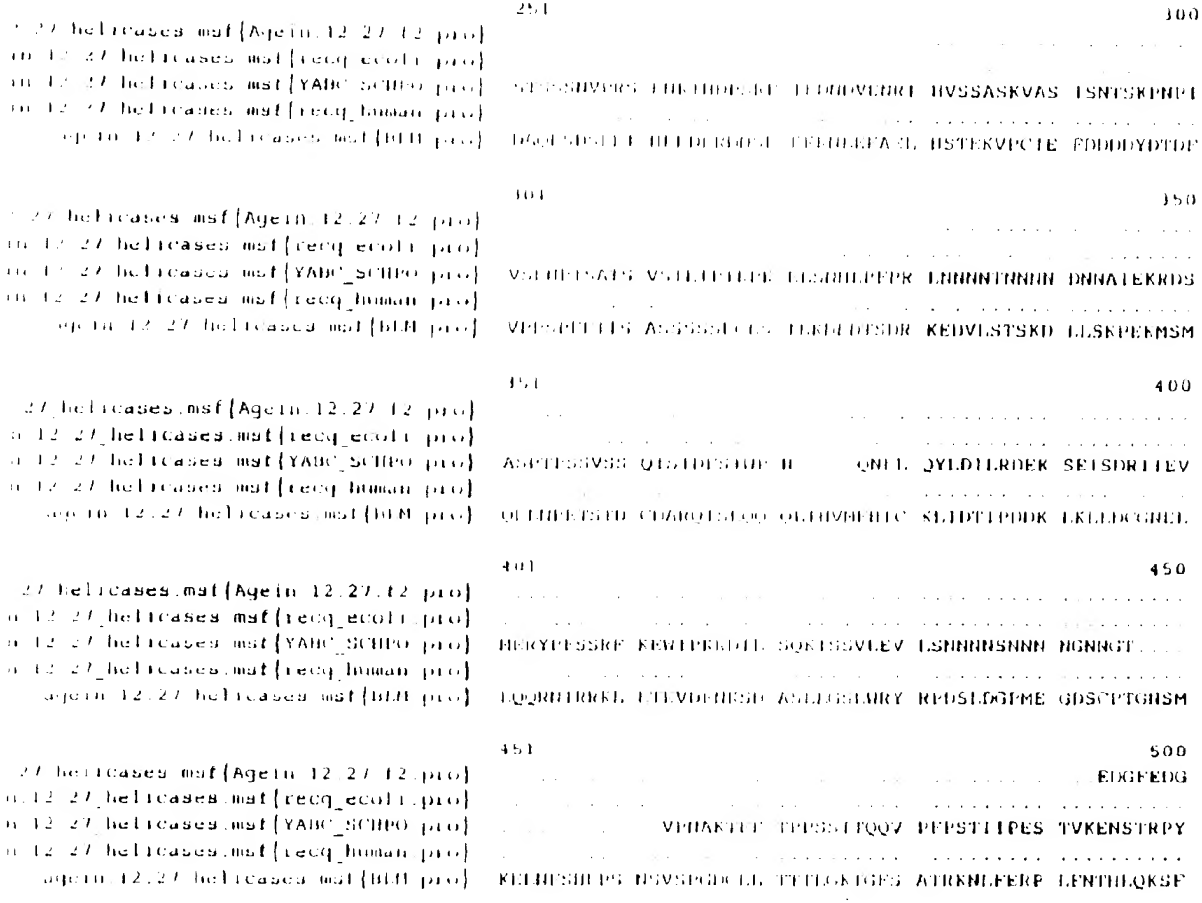


FIGURE 4C

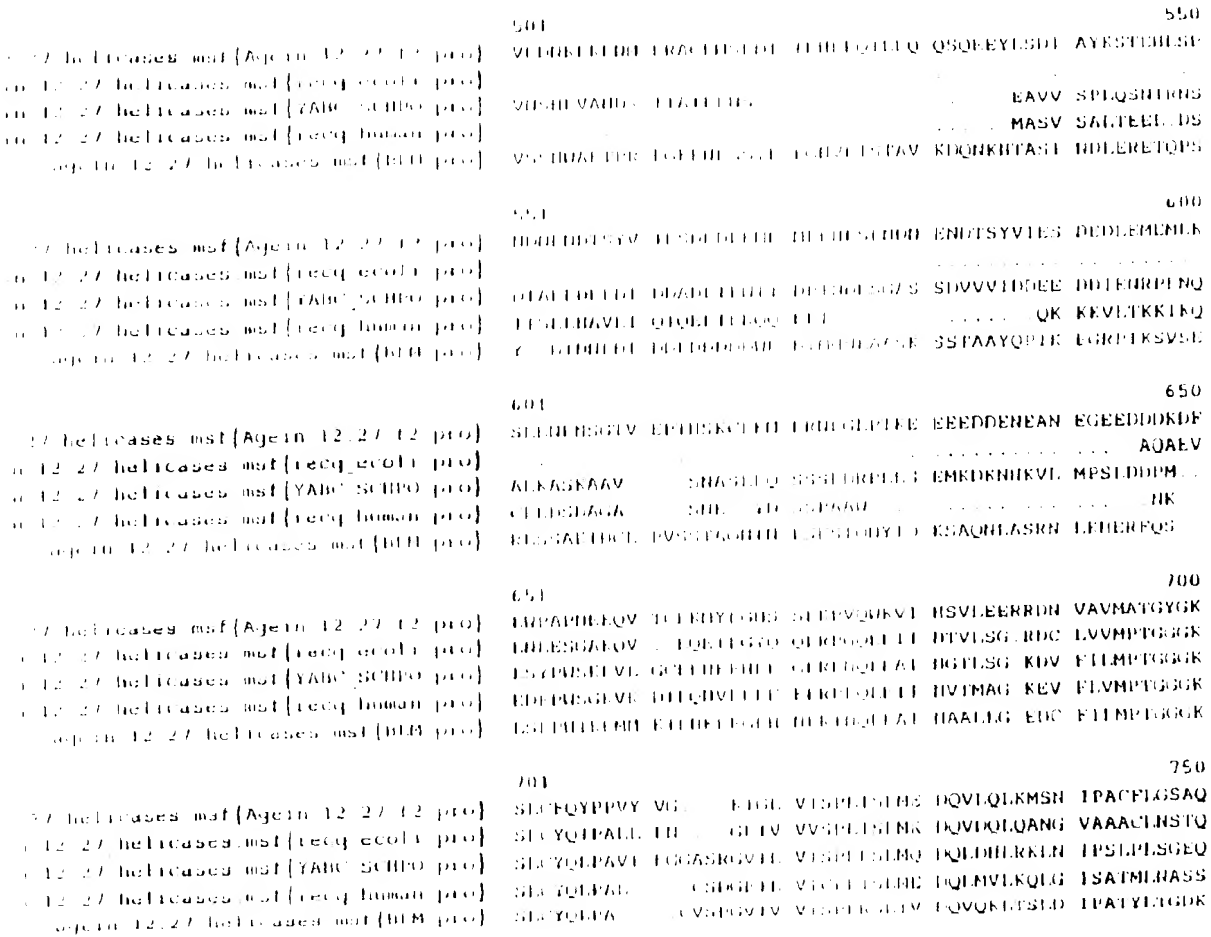


FIGURE 4D

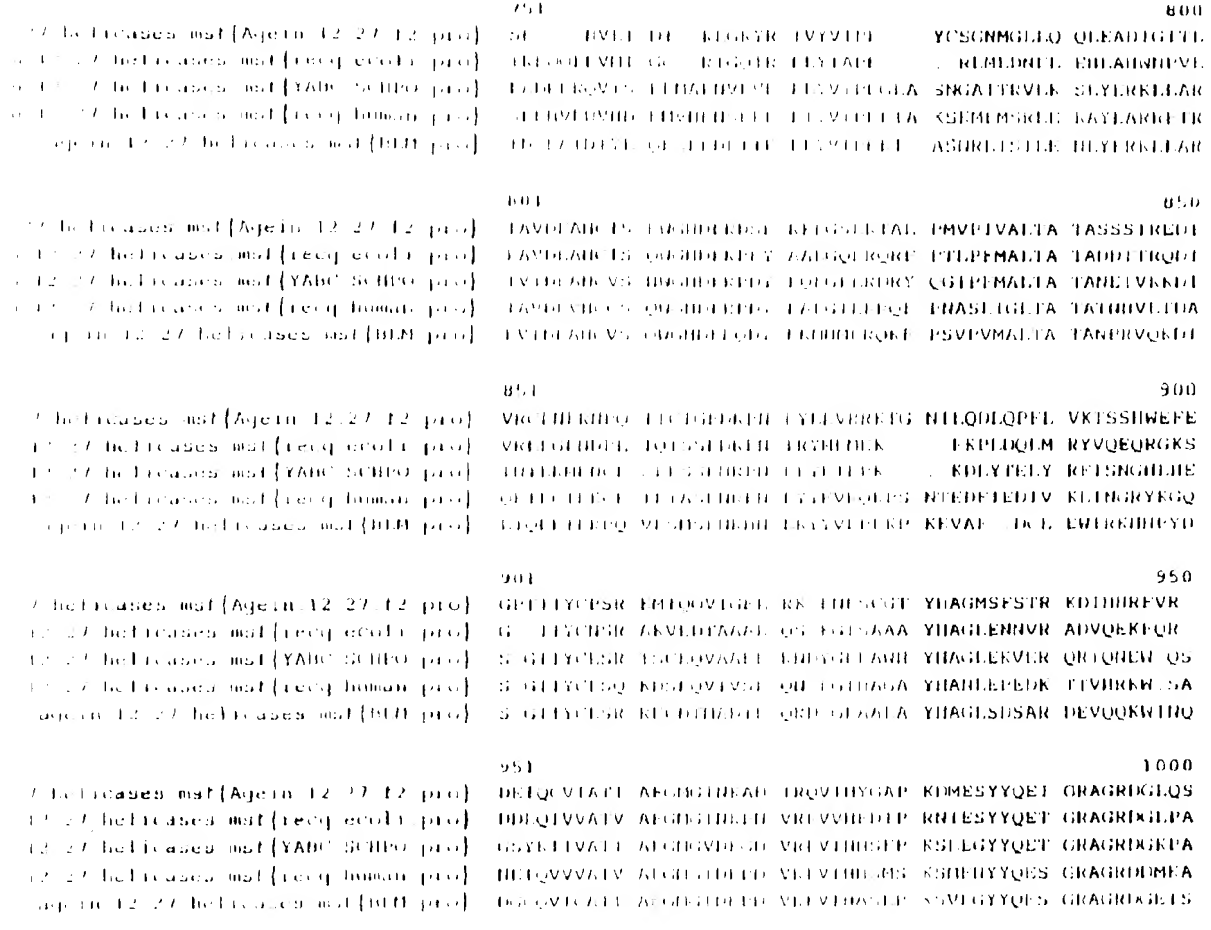


FIGURE 4E

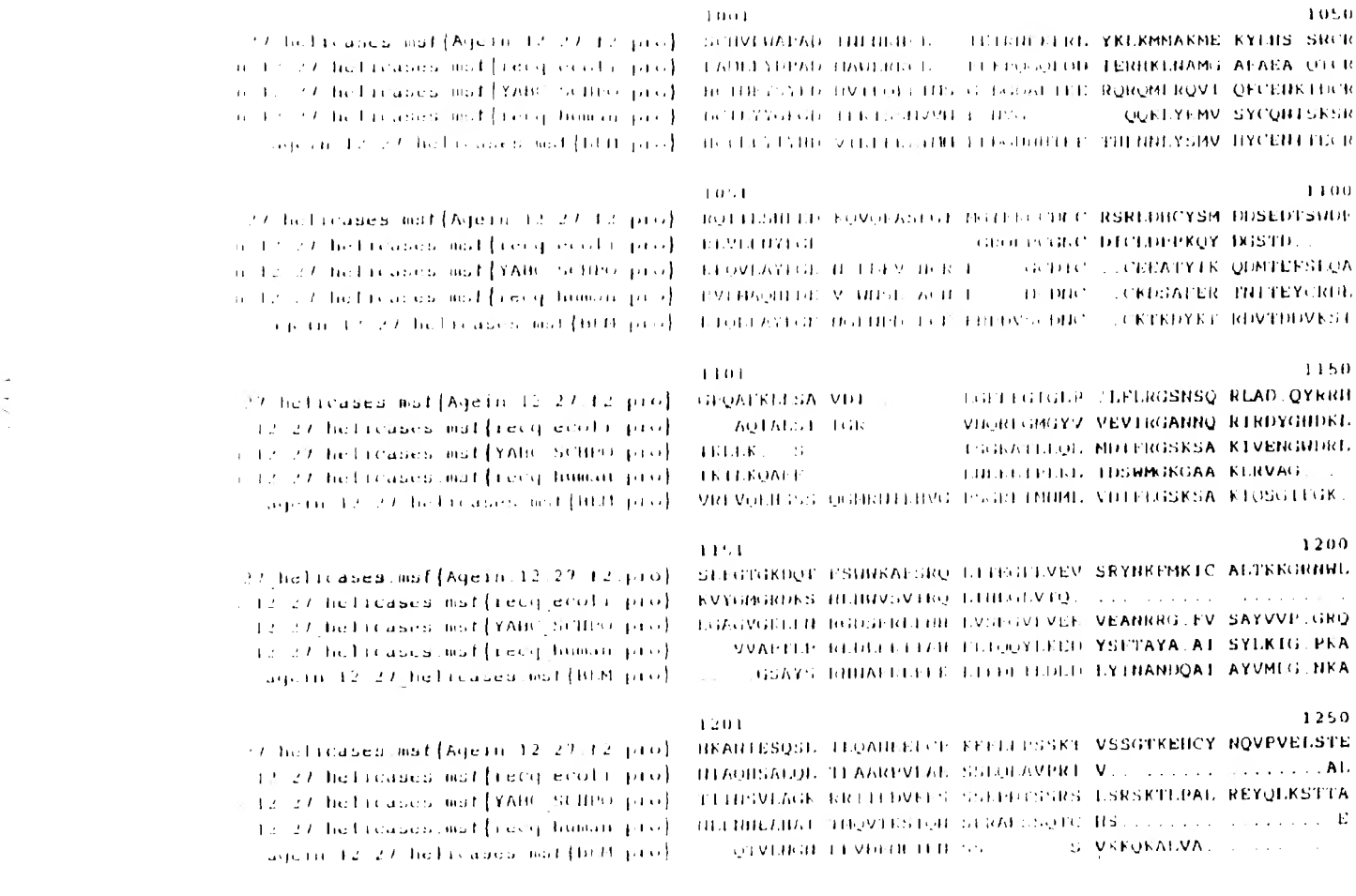


FIGURE 4F

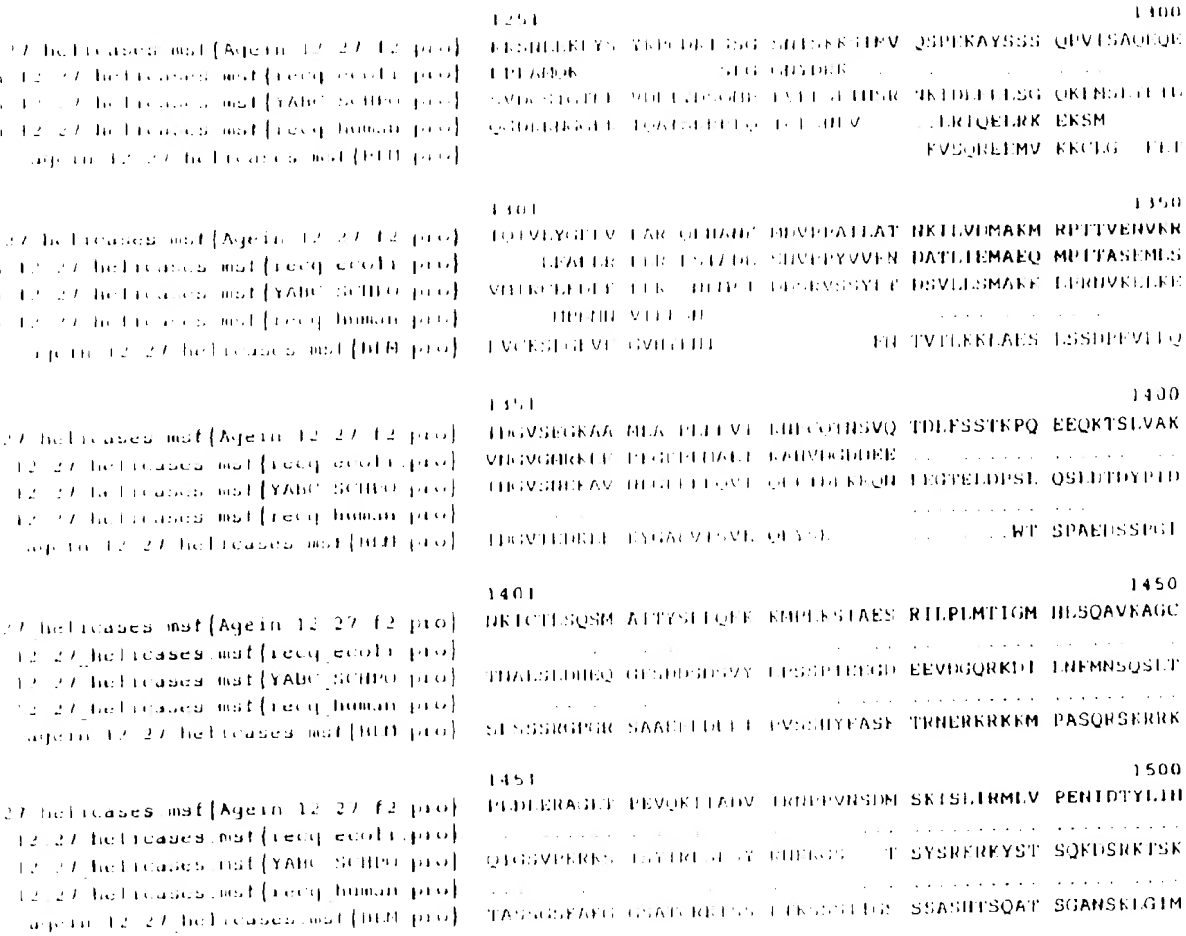


FIGURE 4G

FIGURE 5A

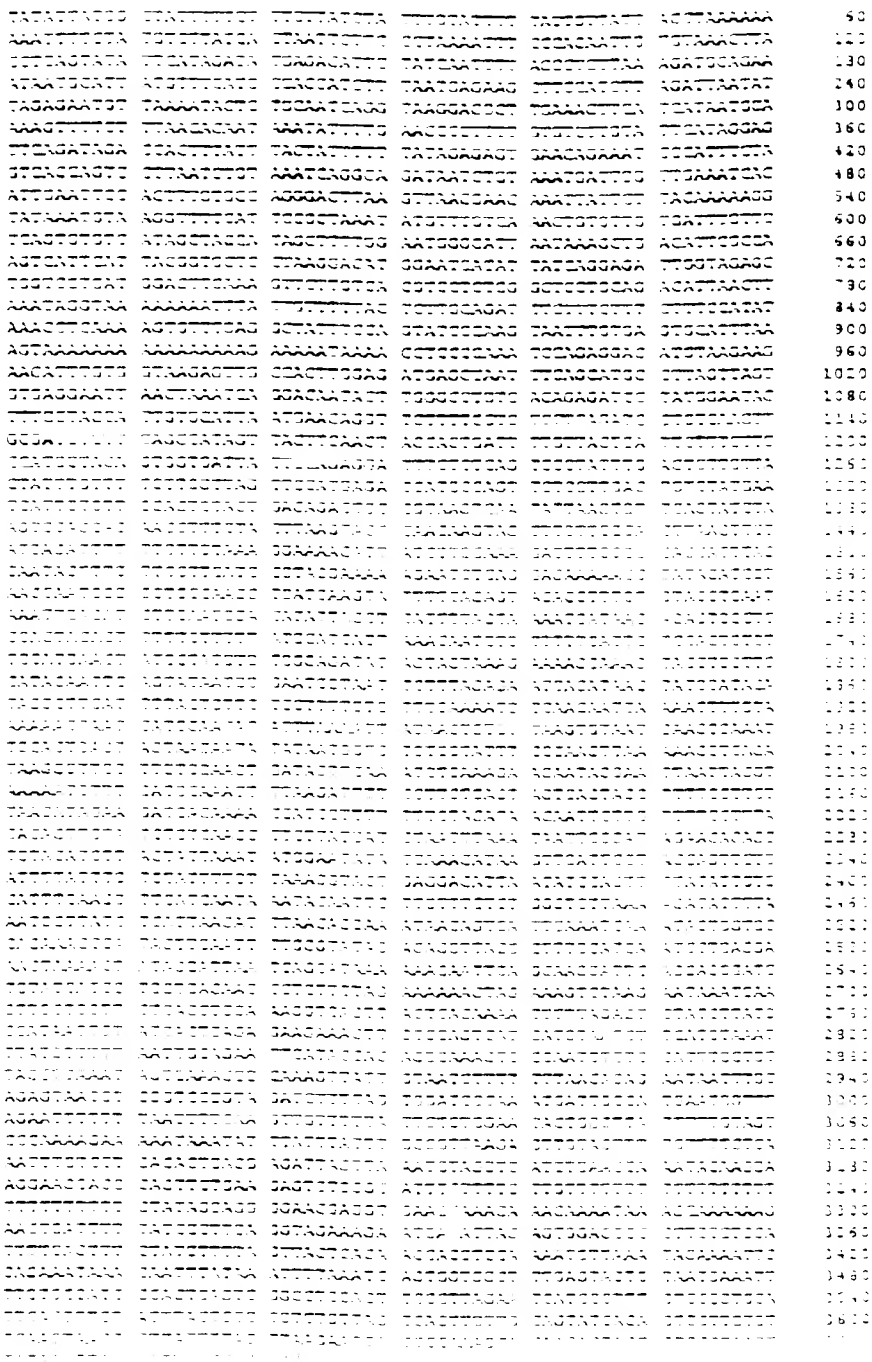


FIGURE 5B

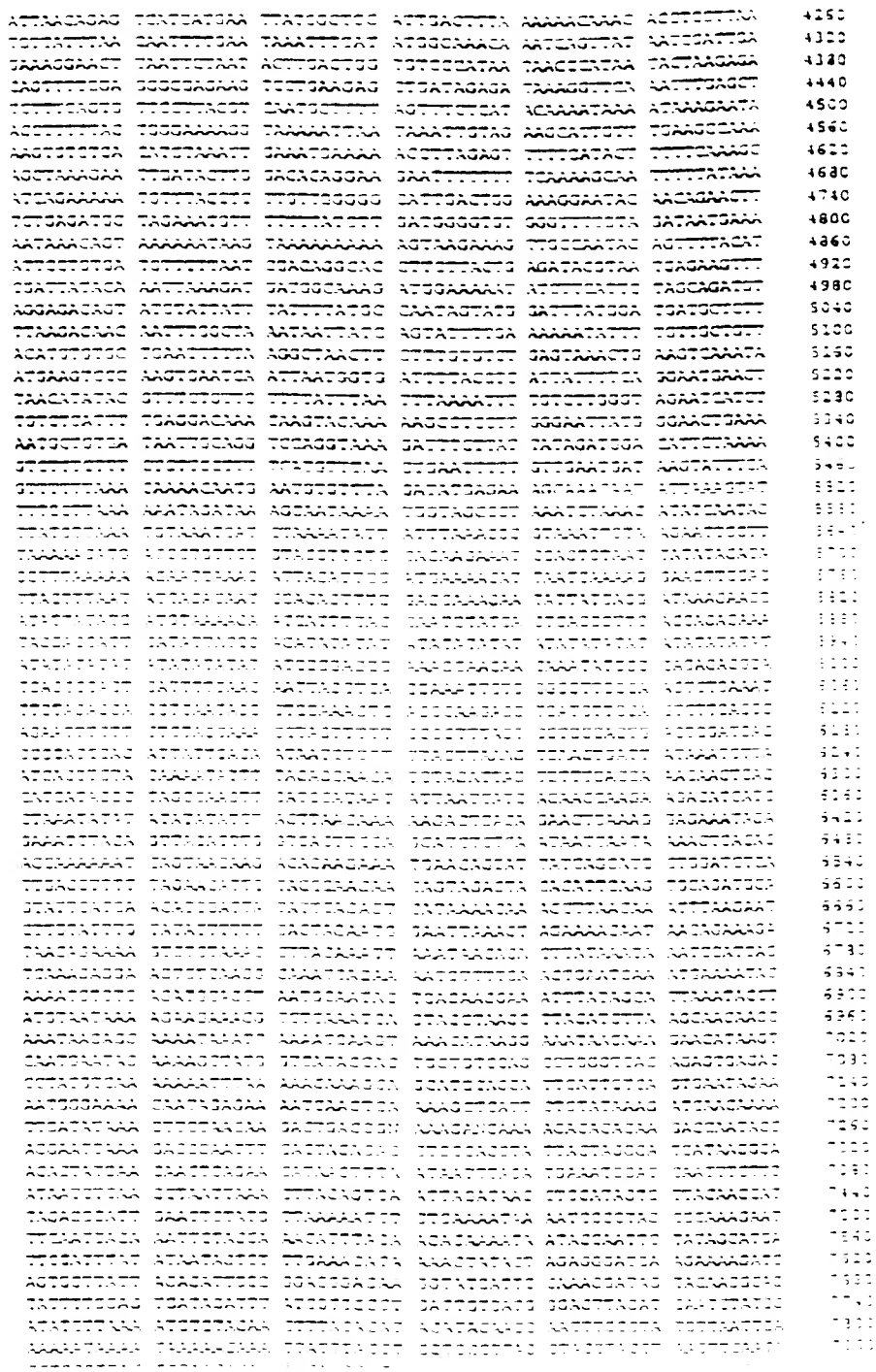


FIGURE 5C

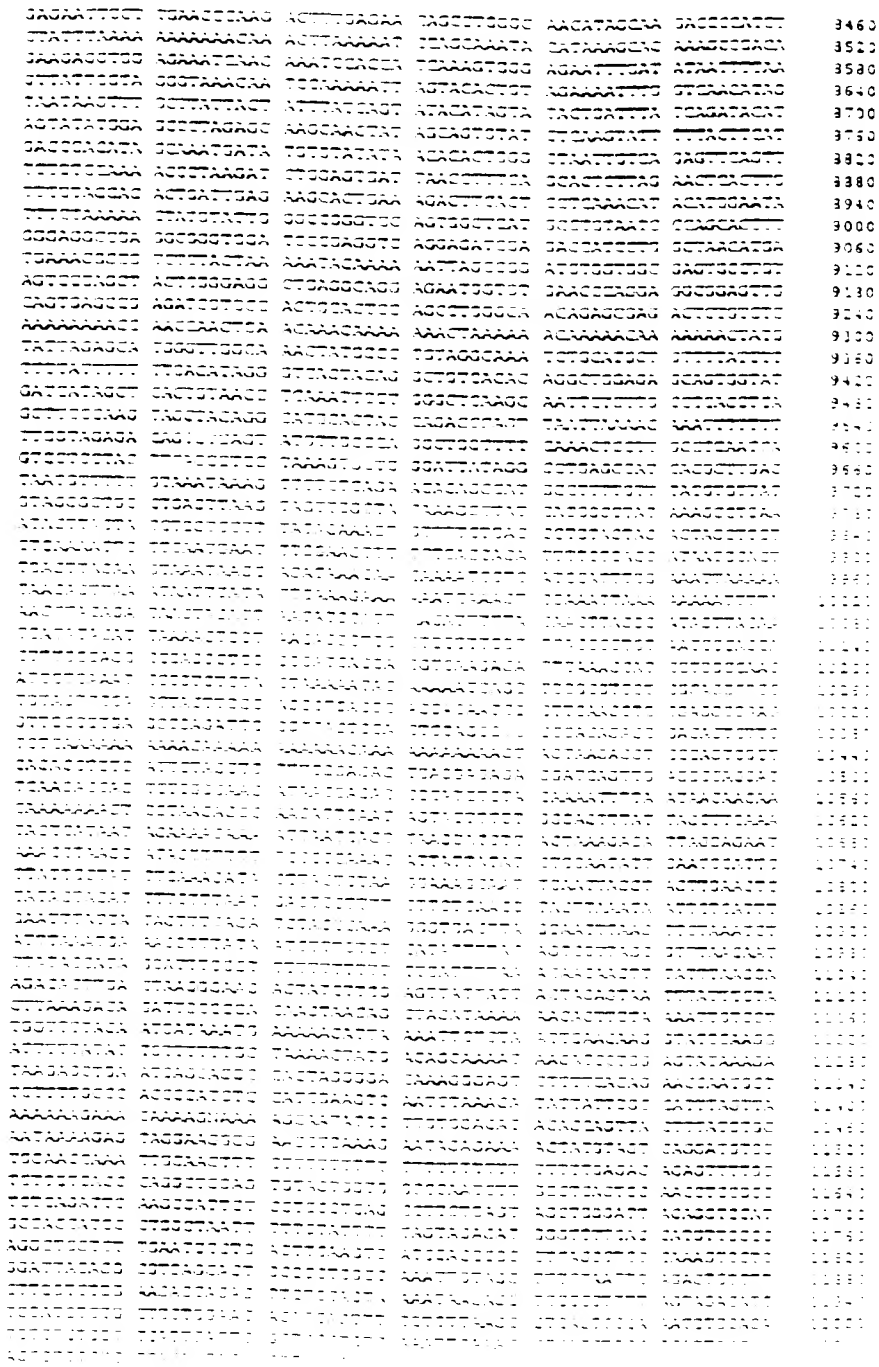


FIGURE 5D

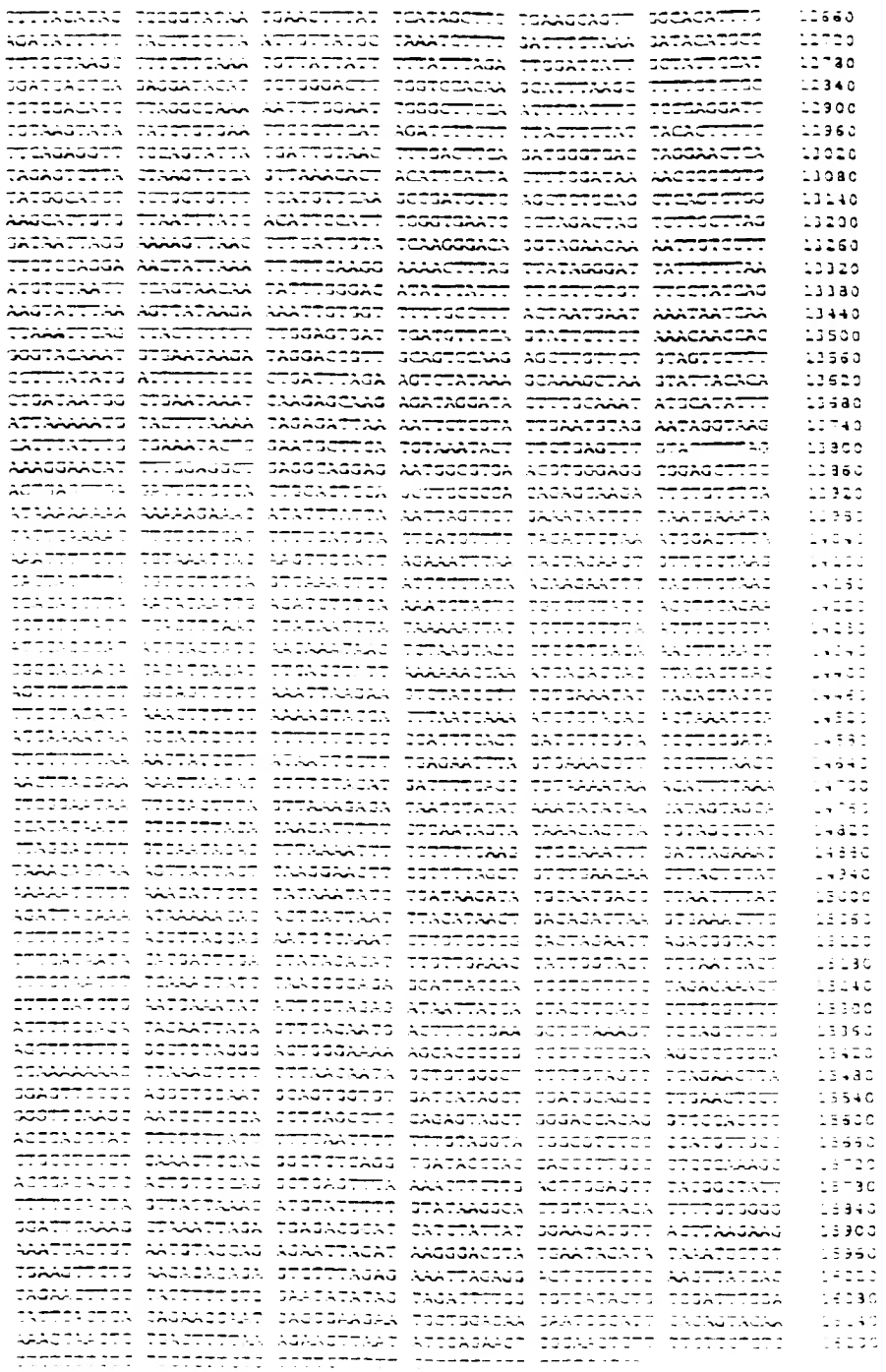


FIGURE 5E

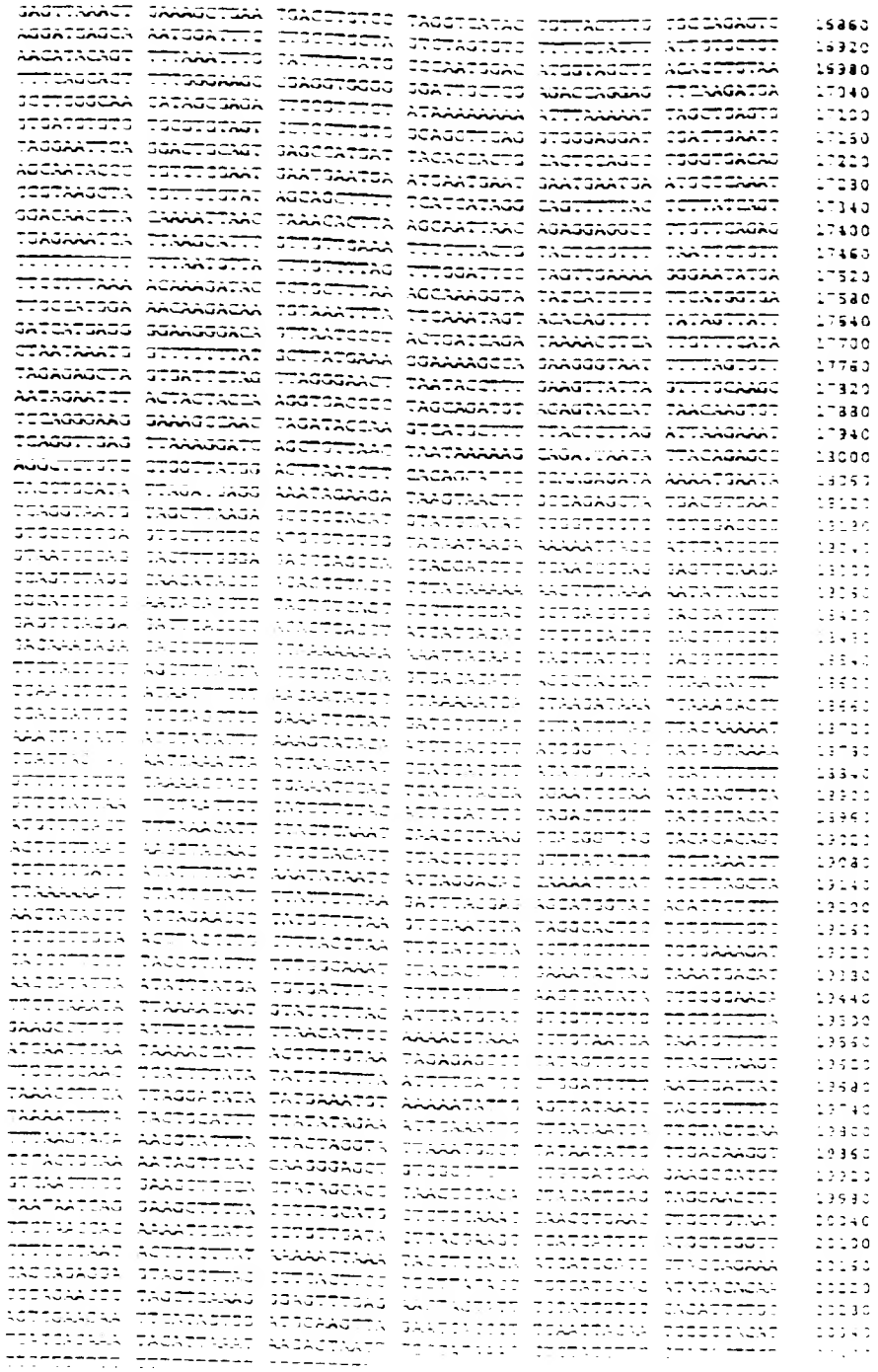


FIGURE 5F

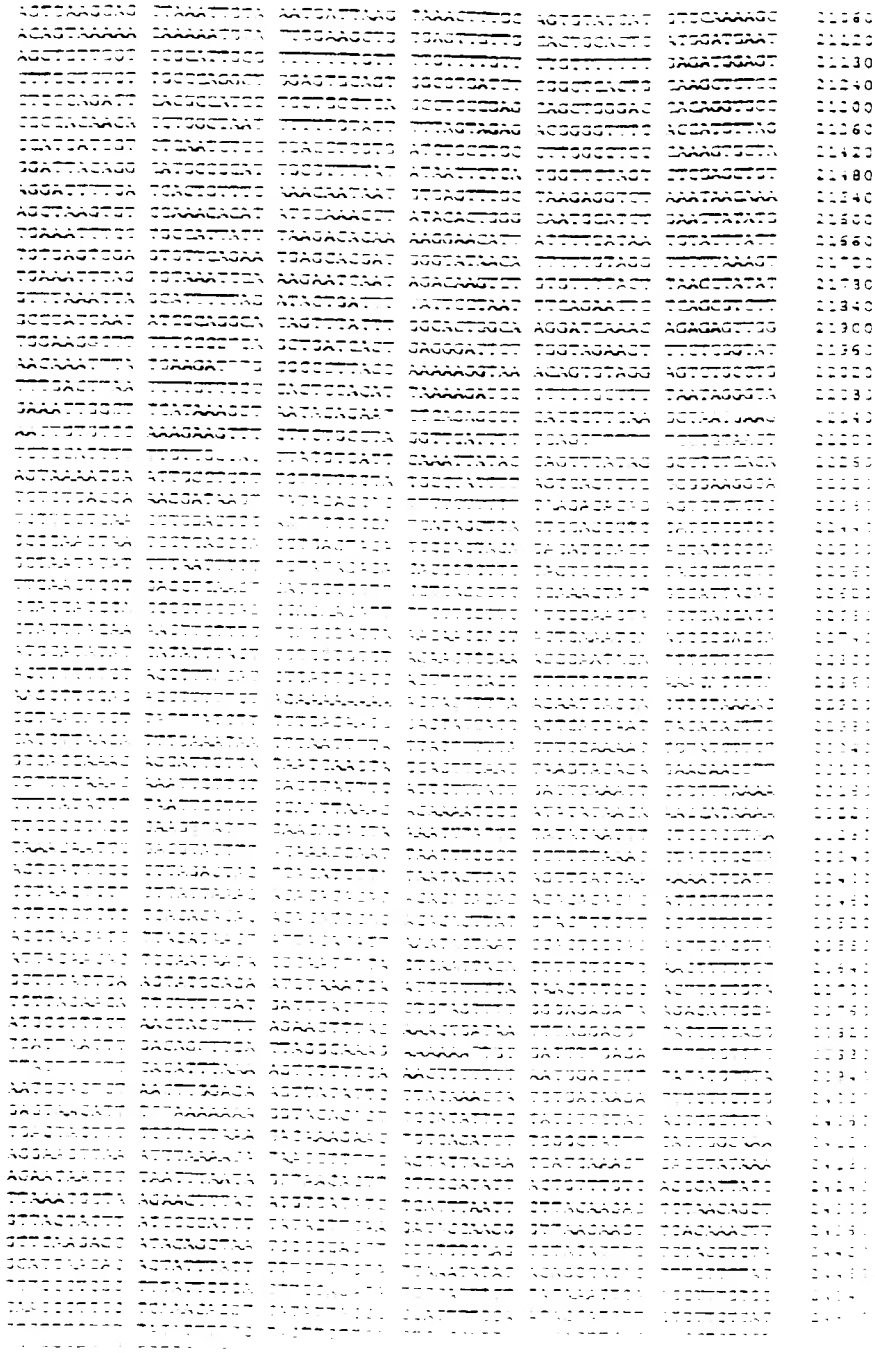


FIGURE 5G

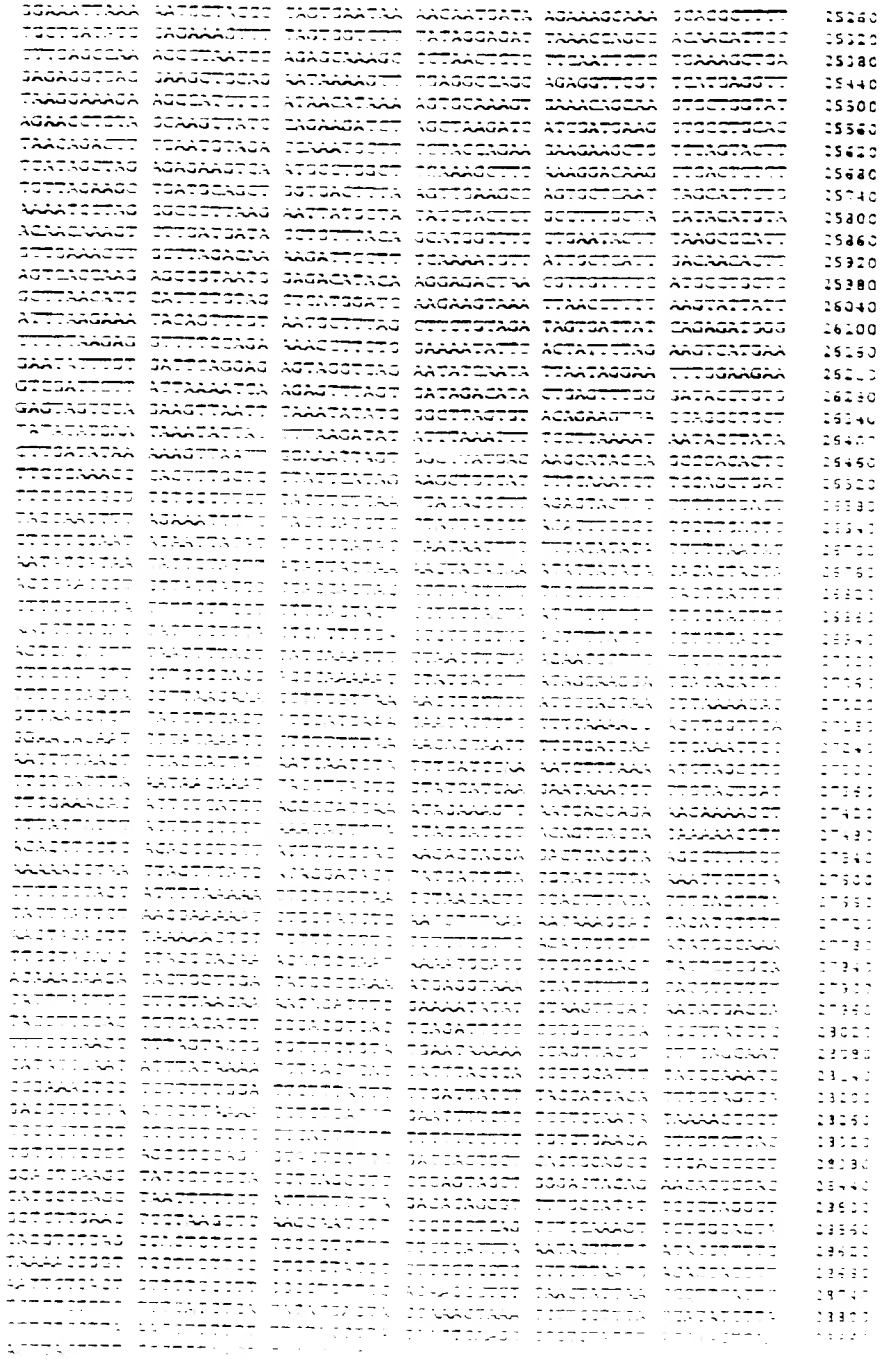


FIGURE 5H

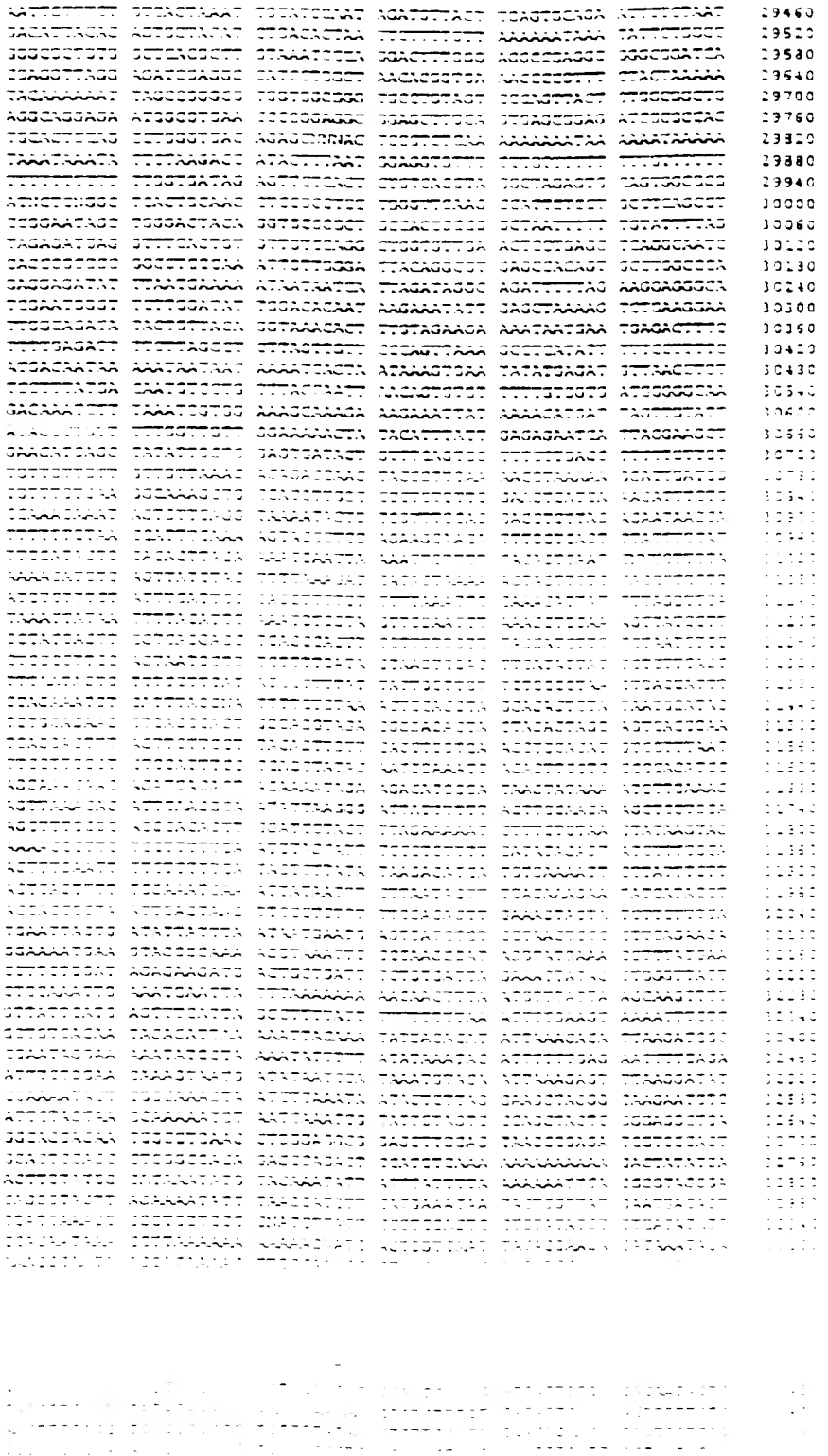


FIGURE 5I

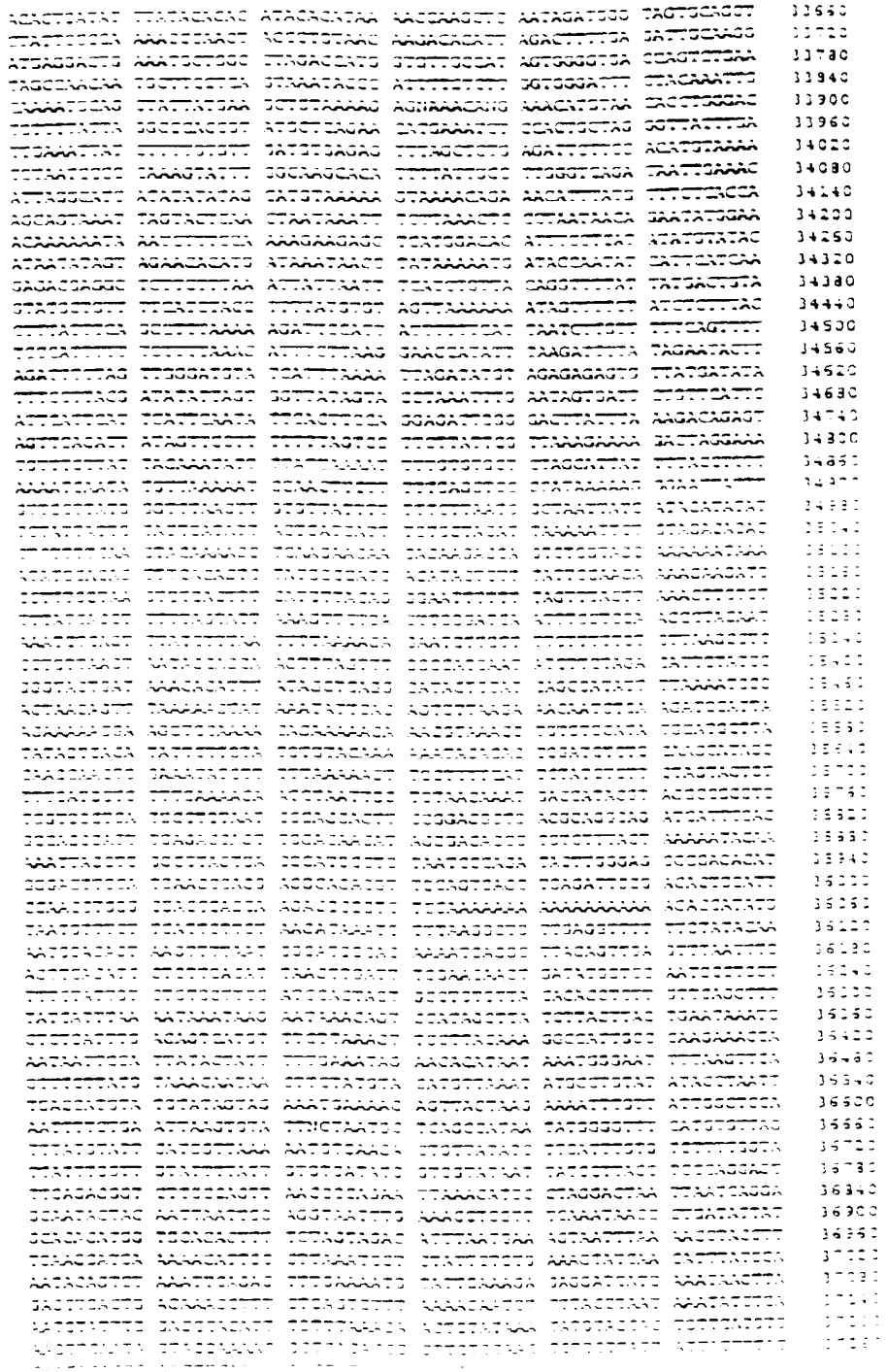


FIGURE 5J

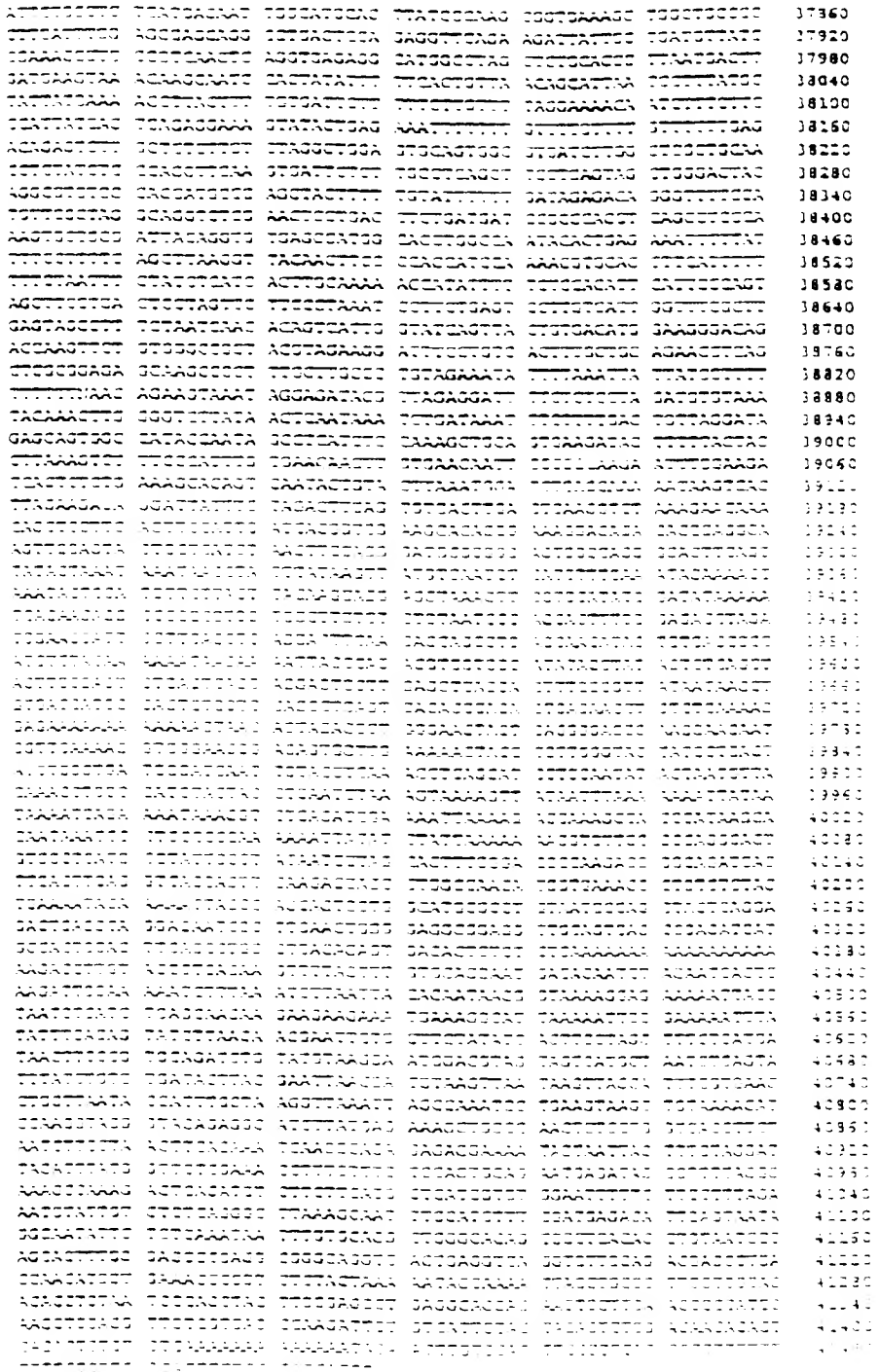


FIGURE 5K

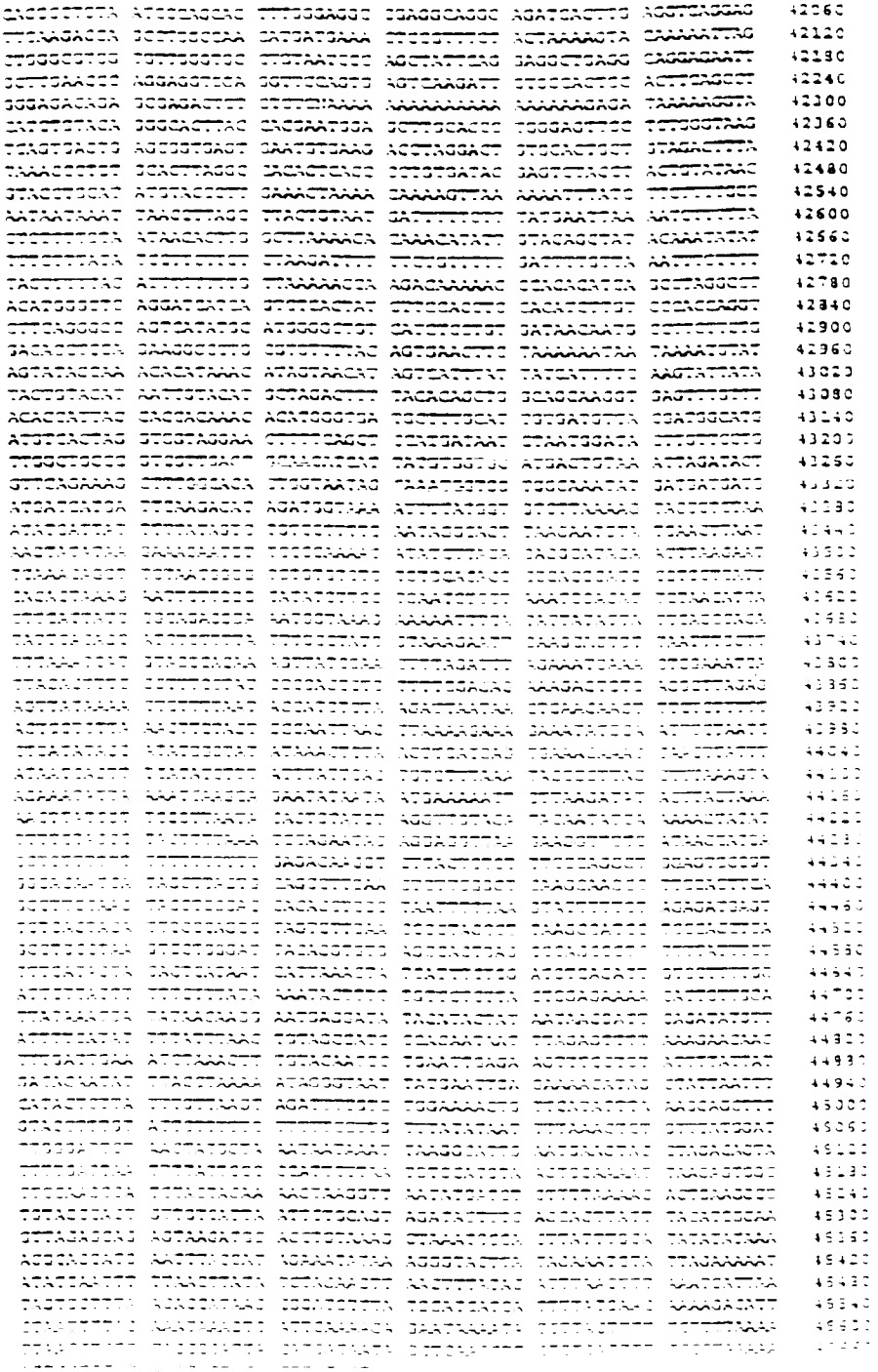


FIGURE 5L

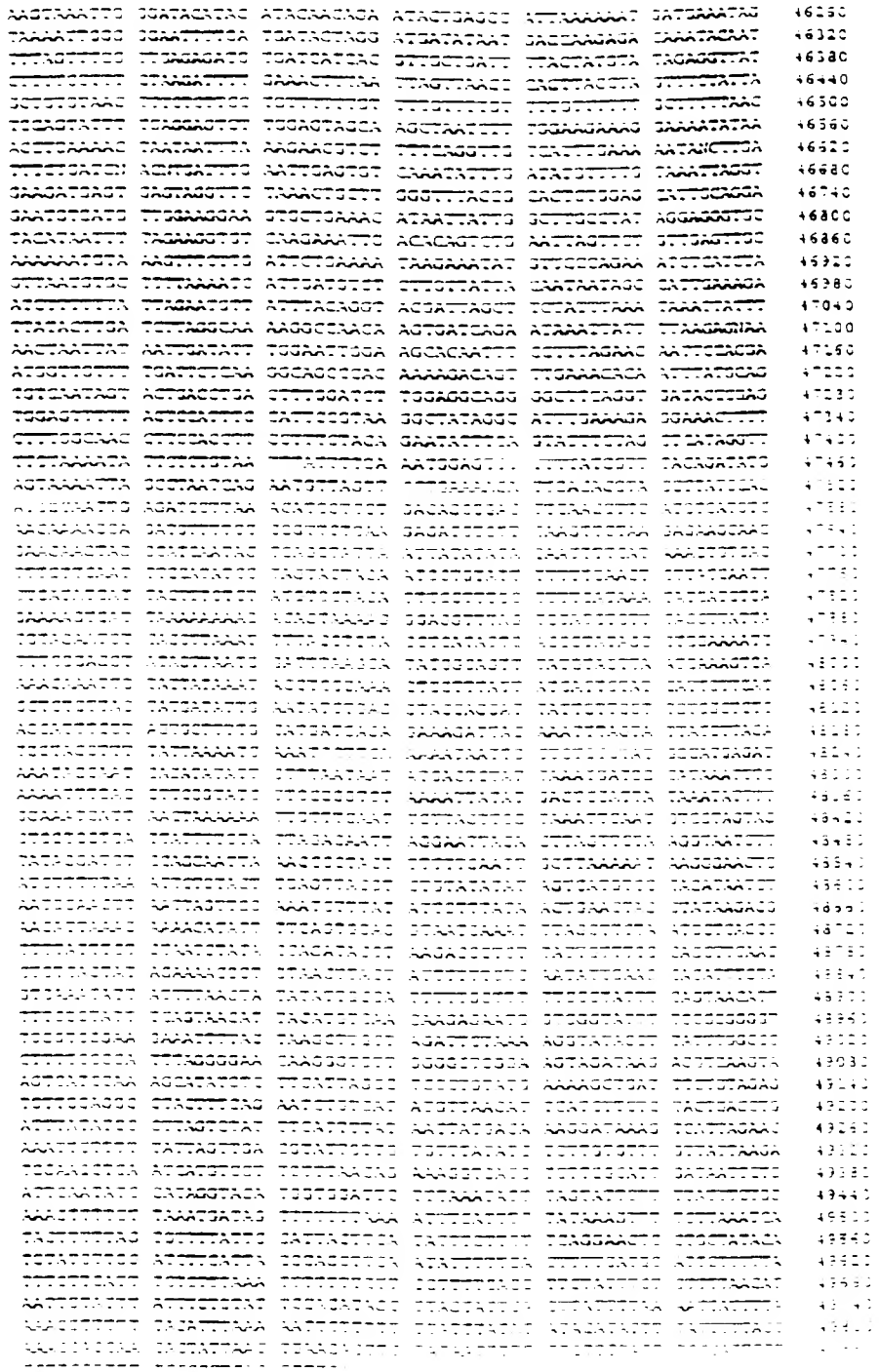


FIGURE 5M

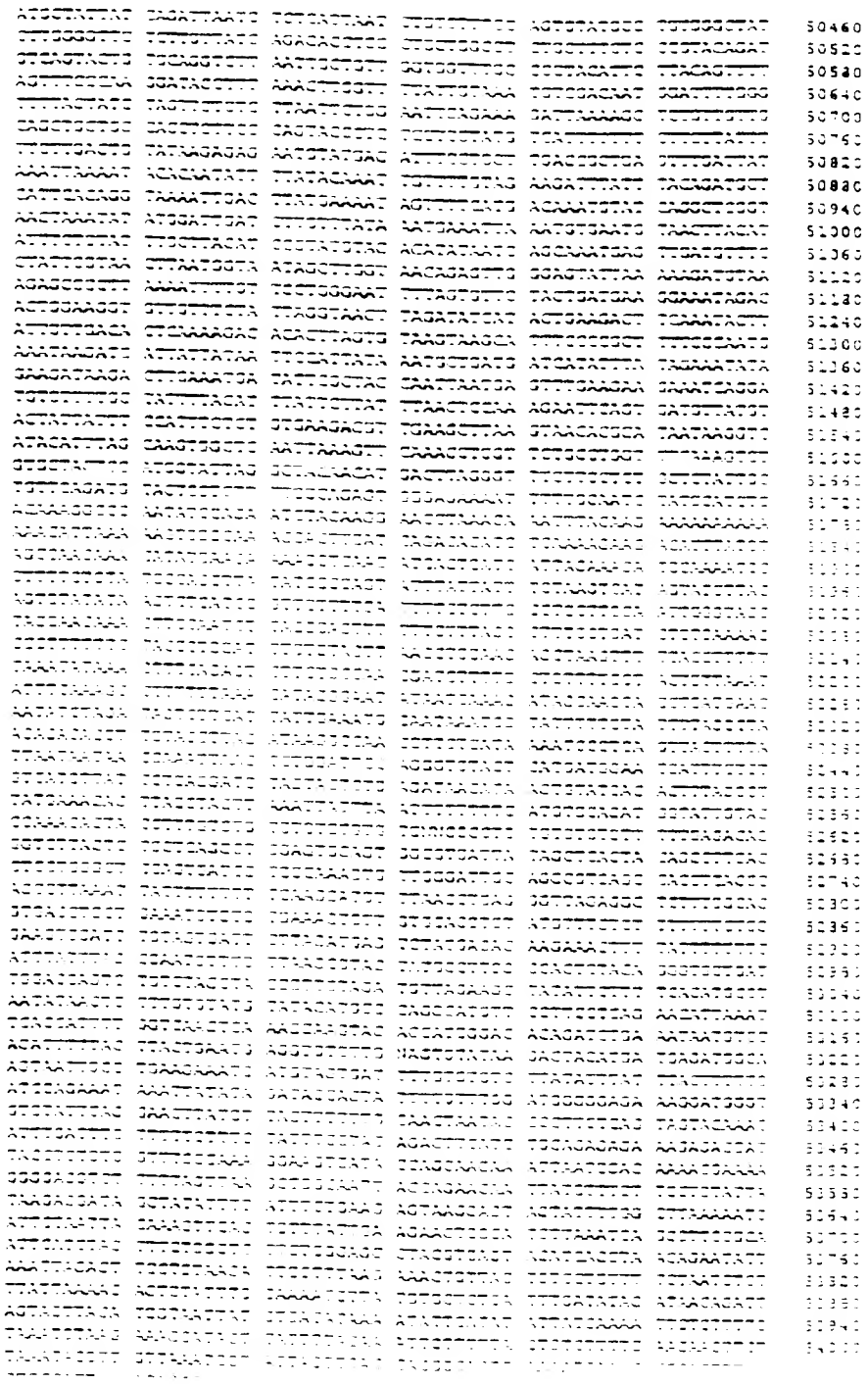


FIGURE 5N

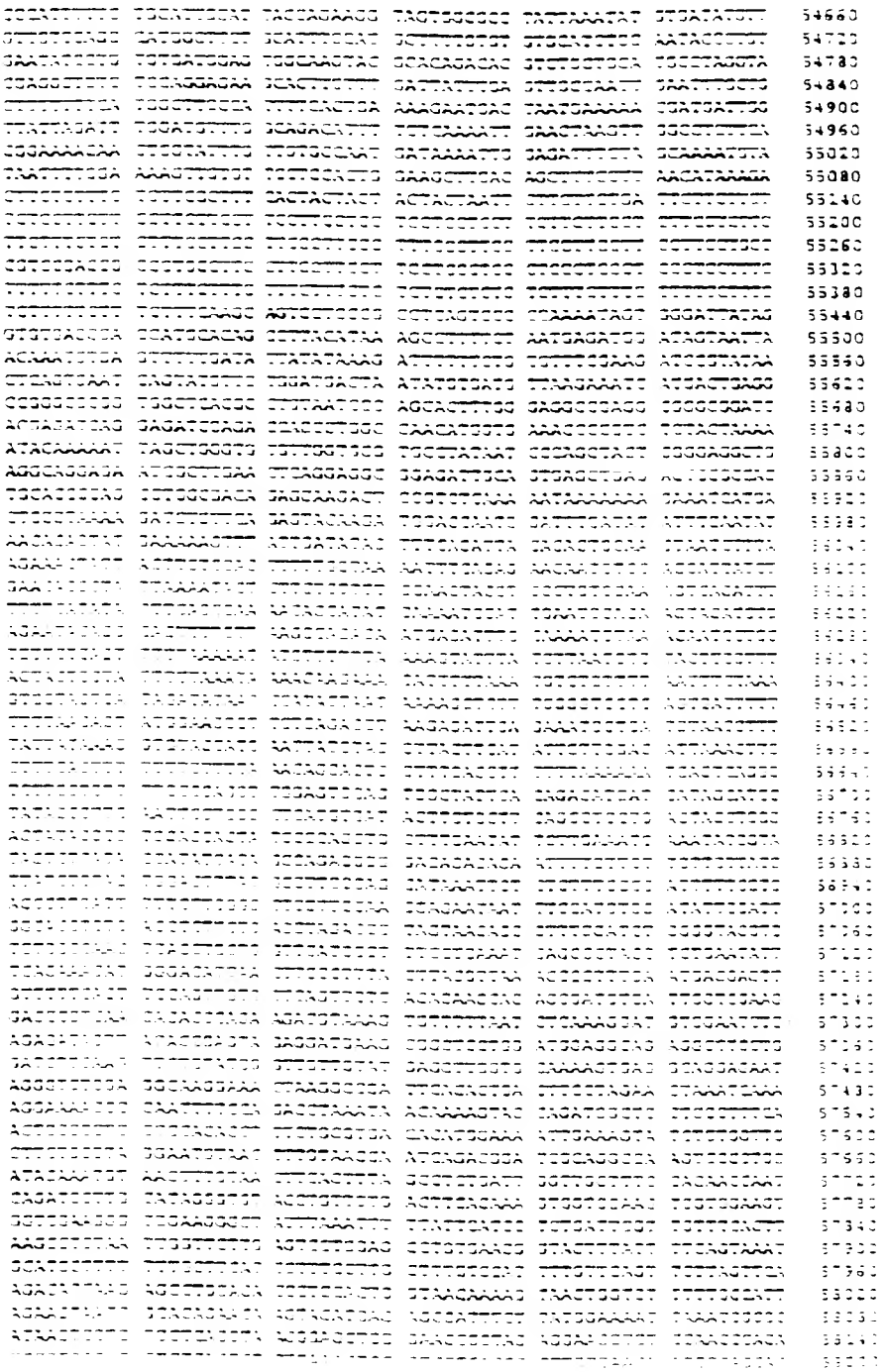


FIGURE 50

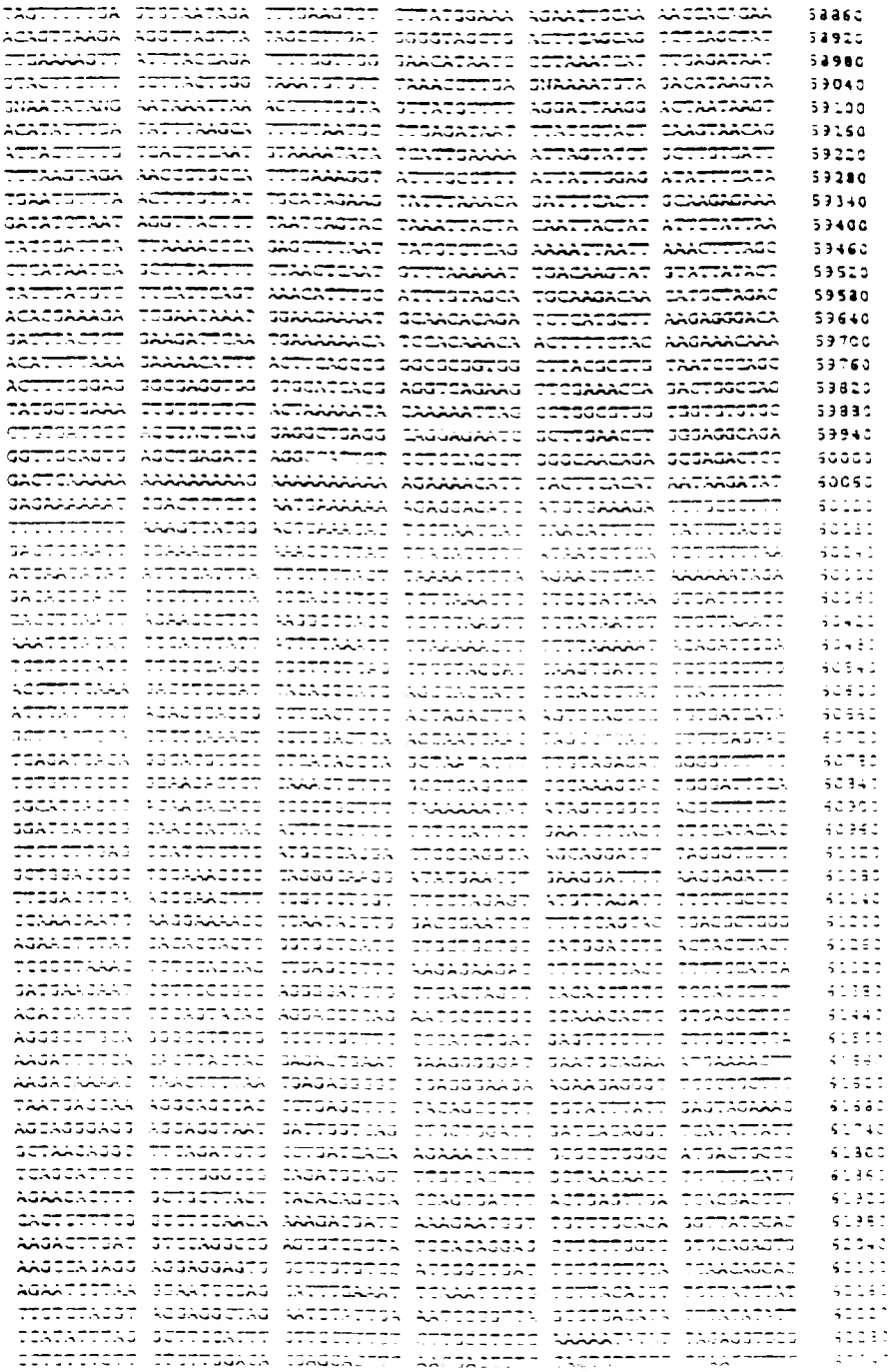


FIGURE 5P

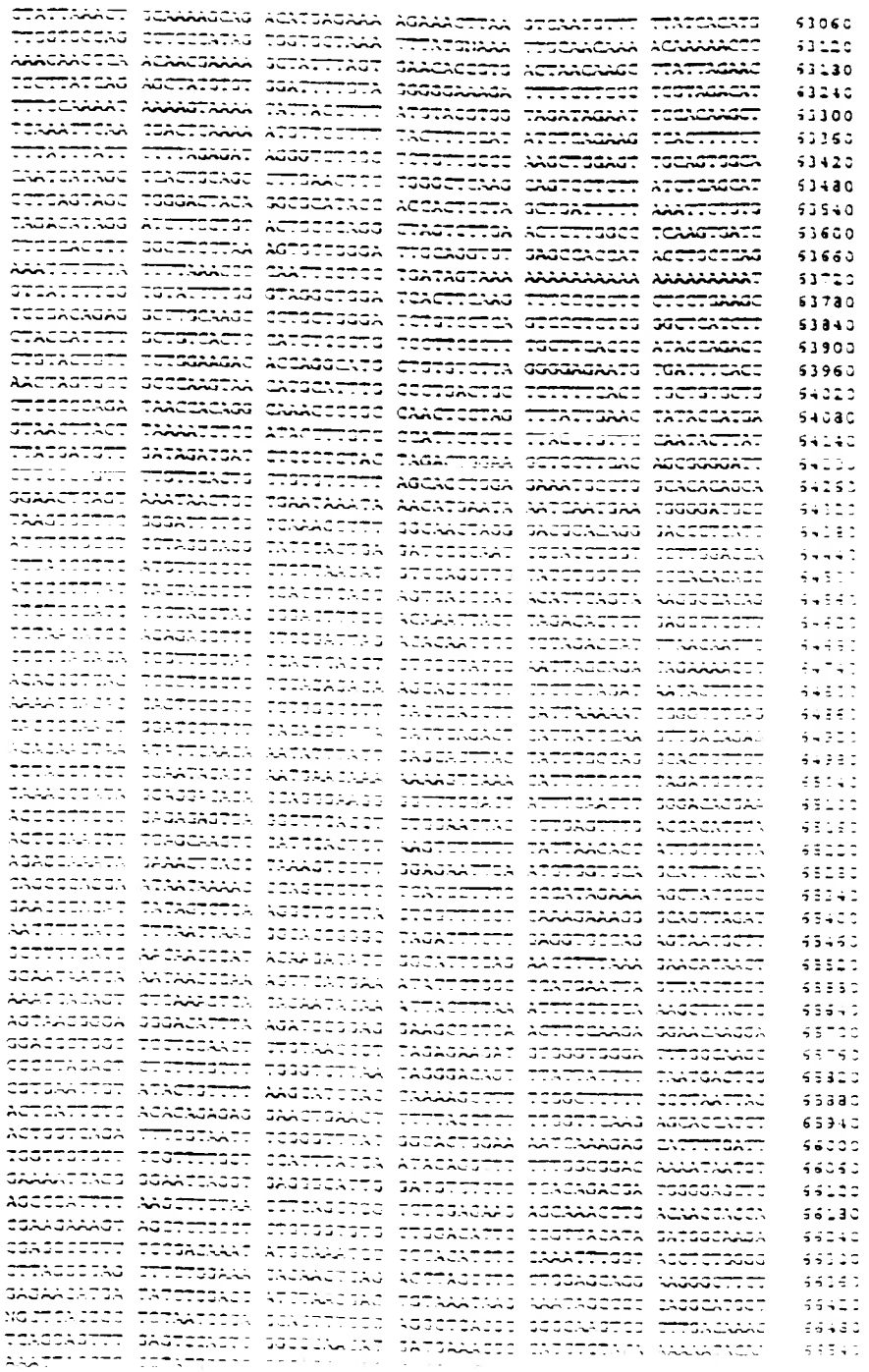


FIGURE 5Q

FIGURE 5R

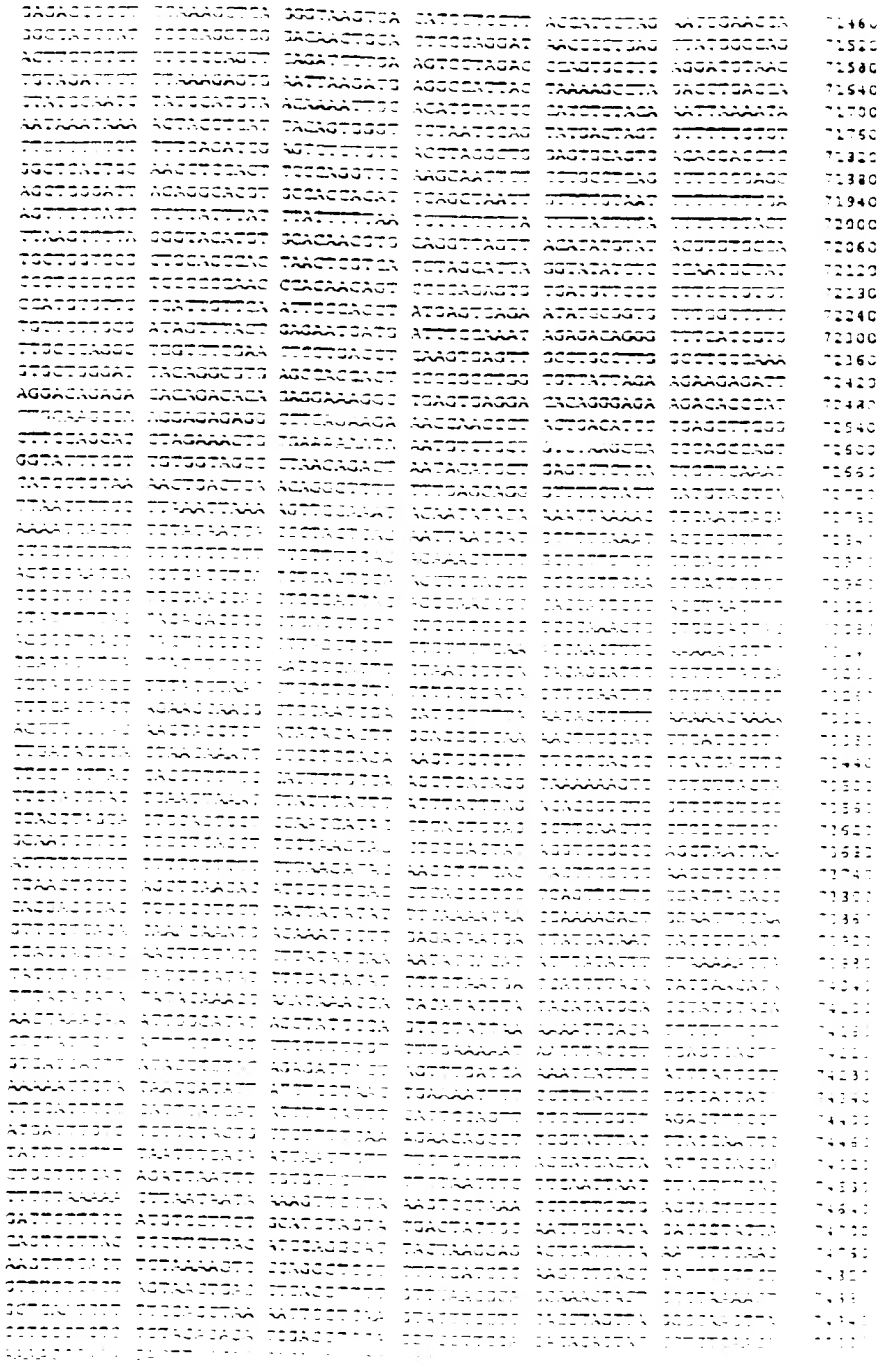


FIGURE 5S

FIGURE 5T

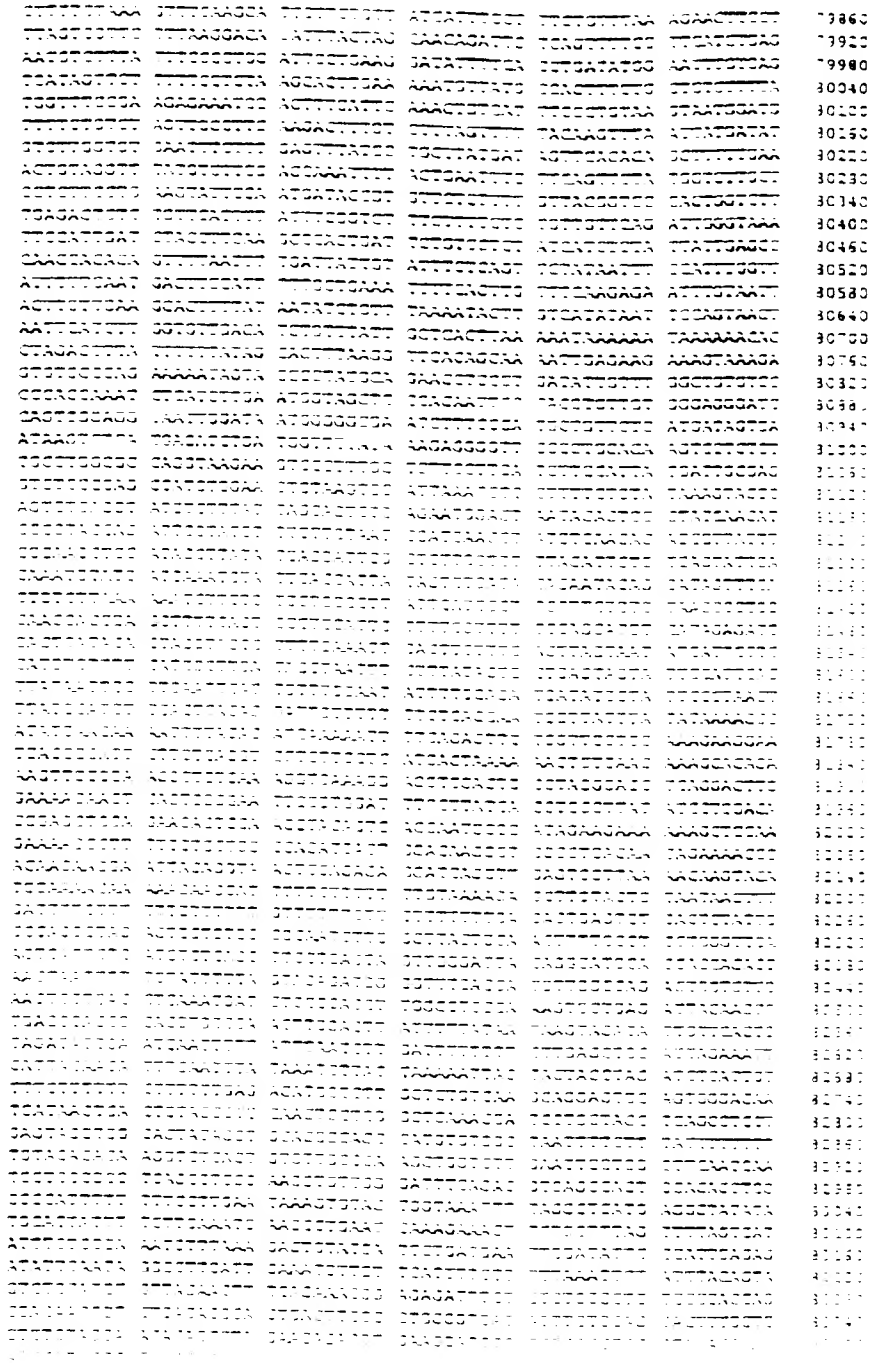


FIGURE 5U

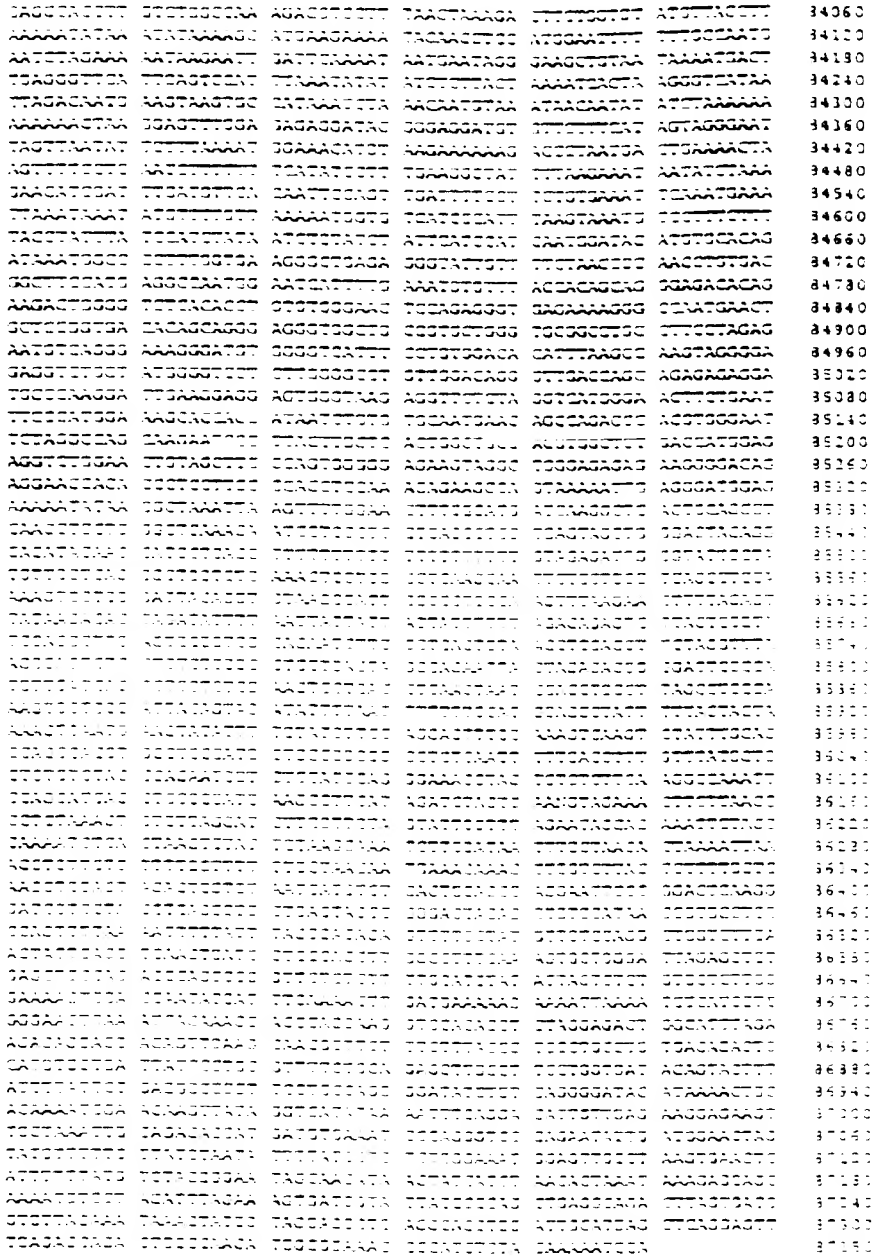


FIGURE 6

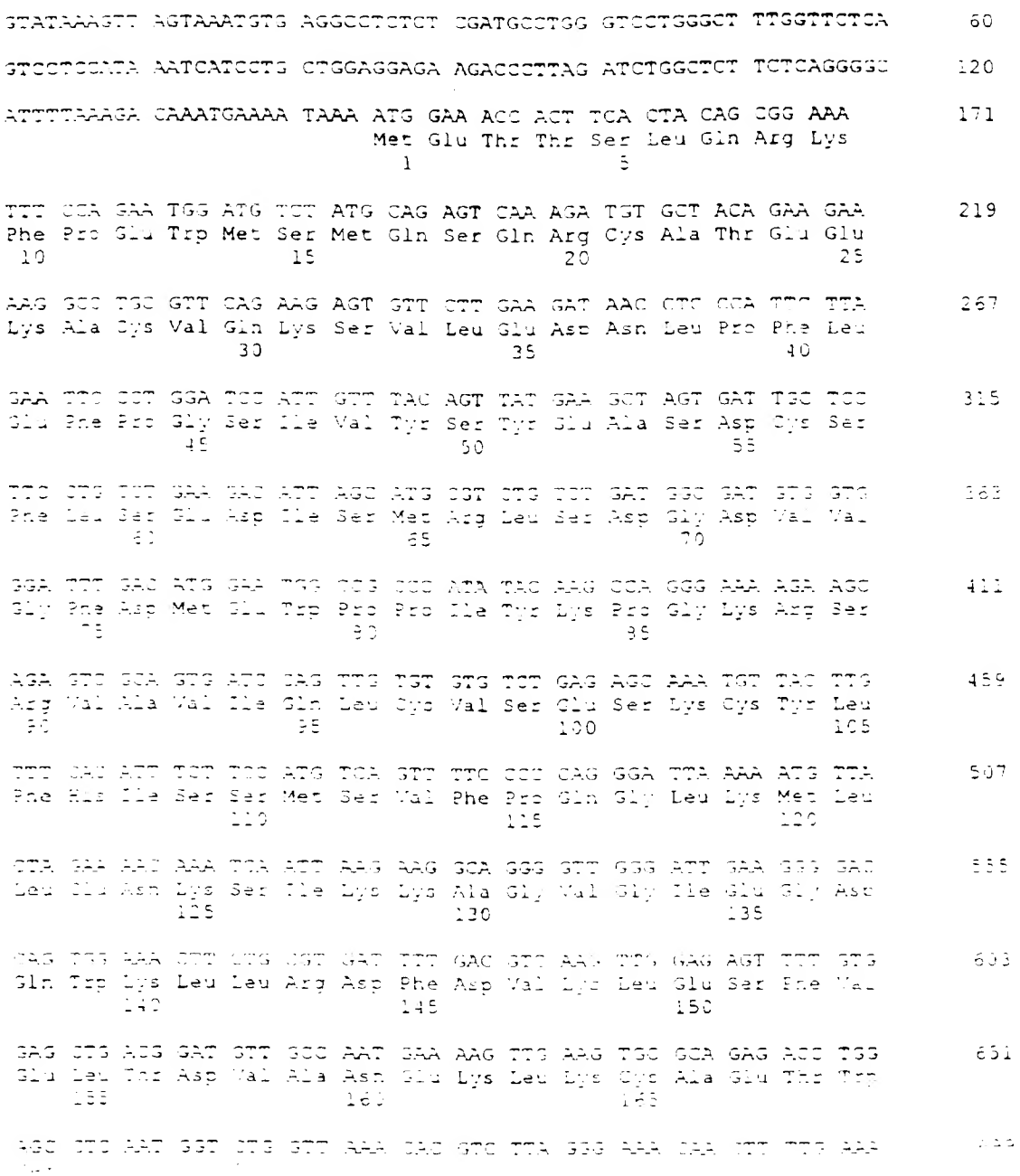

GTATAAAGTT AGTAAATGTG AGGCCTCTCT CGATGCCTGG GTCCTGGGCT TTGGTTCTCA 60
GTCCTCCNTA AATCATCCTG CTGGAGGAGA AGACCCTTAG ATCTGGCTCT TCTCAGGGGC 120
ATTTTAAAGA CAAATGAAAA TAAA ATG GAA ACC ACT TCA CTA CAG CGG AAA 171
Met Glu Thr Thr Ser Leu Gln Arg Lys
1 5
TTT CCA GAA TGG ATG TCT ATG CAG AGT CAA AGA TGT GCT ACA GAA GAA 219
Phe Pro Glu Trp Met Ser Met Gln Ser Gln Arg Cys Ala Thr Glu Glu
10 15 20 25
AAG GCC TGC GTT CAG AAG AGT GTT CTT GAA GAT AAC CTC CCA TTC TTA 267
Lys Ala Cys Val Gln Lys Ser Val Leu Glu Asp Asn Leu Pro Phe Leu
30 35 40
GAA TTC CCT GGA TCC ATT GTT TAC AGT TAT GAA GCT AGT GAT TGC TCC 315
Glu Phe Pro Gly Ser Ile Val Tyr Ser Tyr Glu Ala Ser Asp Cys Ser
45 50 55
TTC CTG TCT GAA GAC ATT AGC ATG CGT CTG TCT GAT GGC GAT GTG GTG 363
Phe Leu Ser Glu Asp Ile Ser Met Arg Leu Ser Asp Gly Asp Val Val
60 65 70
GGA TTT GAC ATG GAA TGG CCG CCC ATA TAC AAG CCA GGG AAA AGA AGC 411
Gly Phe Asp Met Glu Trp Pro Pro Ile Tyr Lys Pro Gly Lys Arg Ser
75 80 85
AGA GTC GCA GTG ATC CAG TTG TGT GTG TCT GAG AGC AAA TGT TAC TTG 459
Arg Val Ala Val Ile Gln Leu Cys Val Ser Glu Ser Lys Cys Tyr Leu
90 95 100 105
TTT GAC ATT TCT TCC ATG TCA GTT TTC CCC CAG GGA TTA AAA ATG TTA 507
Phe His Ile Ser Ser Met Ser Val Phe Pro Gln Gly Leu Lys Met Leu
110 115 120
CTA GAA AAC AAA TCA ACT AAG AAG GCA GGG GTT GGG ATT GAA GGG GAC 555
Leu Glu Asn Lys Ser Ile Lys Lys Ala Gly Val Gly Ile Glu Gly Asp
125 130 135
CAG TGG AAA CTT CTG CGT GAT TTT GAC GTT AAA CTG GAG AGT TTT GTG 603
Gln Trp Lys Leu Leu Arg Asp Phe Asp Val Lys Leu Glu Ser Phe Val
140 145 150
GAG CTG ACG GAT GTT GCC AAT GAA AAG TTG AAG TGC GCA GAG ACC TGG 651
Glu Leu Thr Asp Val Ala Asn Glu Lys Leu Lys Cys Ala Glu Thr Trp
155 160 165
AGC CTG AAT GGT CTG GTT AAA GAC GTC TTA GGG AAA CAA CTT TTT AAA

FIGURE 6 (CONT.)

```
GAC CAG AAA CTG TAT GCA GCC ACT GAT GCT TAT GCT GGT CTT ATC ATC      795
Asp Gln Lys Leu Tyr Ala Ala Thr Asp Ala Tyr Ala Gly Leu Ile Ile
            205                 210                 215

TAT CAA AAA TTA GGA AAT TTG GGT GAT ACT GCG CAA GTG TTT GCT CTA      843
Tyr Gln Lys Leu Gly Asn Leu Gly Asp Thr Ala Gln Val Phe Ala Leu
        220                 225                 230

AAT AAA GCA GAG GAA AAC CTA CCT CTG GAG ATG AAG AAA CAG TTG AAT      891
Asn Lys Ala Glu Glu Asn Leu Pro Leu Glu Met Lys Lys Gln Leu Asn
    235                 240                 245

TCA ATC TCC GAA GAA ATG AGG GAC CTA GCC AAT CGT TTT CCT GTC ACT      939
Ser Ile Ser Glu Glu Met Arg Asp Leu Ala Asn Arg Phe Pro Val Thr
250                 255                 260                 265

TGC AGA AAT TTG GAA ACT CTC CAG AGG GTT CCT GTA ATA TTG AAG AGT      987
Cys Arg Asn Leu Glu Thr Leu Gln Arg Val Pro Val Ile Leu Lys Ser
                270                 275                 280

ATT TCA GAA AAT CTC TGT TCA TTG AGA AAA GTG ATC TGT GGT CCT AGA     1035
Ile Ser Glu Asn Leu Cys Ser Leu Arg Lys Val Ile Cys Gly Pro Thr
            285                 290                 295

AAC ACT GAG ACT AGA CTG AAG CCG GGC AGT AGT TTT AAT TTA CTG TCA     1083
Asn Thr Glu Thr Arg Leu Lys Pro Gly Ser Ser Phe Asn Leu Leu Ser
        300                 305                 310

TCA GAG GAT TCA GCT GCT GCT GGA GAA AAA GAG AAA CAG ATT GGA AAA     1131
Ser Glu Asp Ser Ala Ala Ala Gly Glu Lys Glu Lys Gln Ile Gly Lys
    315                 320                 325

CAT AGT ACT TTT GCT AAA ATT AAA GAA GAA CCA TGG GAC CCA GAA CTT     1179
His Ser Thr Phe Ala Lys Ile Lys Glu Glu Pro Trp Asp Pro Glu Leu
330                 335                 340                 345

GAC AGT TTA GTT AAG GAA GAG GAG GTT GAT GTA TTT AGA AAT GAA GTG     1227
Asp Ser Leu Val Lys Glu Glu Glu Val Asp Val Phe Arg Asn Glu Val
                350                 355                 360

AAG GAA GAA AAA AGT GGA TCT GAA AAT GAA ATA GAA GAC AAT CTG TTG     1275
Lys Glu Glu Lys Ser Gly Ser Glu Asn Glu Ile Glu Asp Asn Leu Leu
            365                 370                 375

AGA GAA GAT ATG GAA AGA ACT TGT GTG ATT CCT AGT ATT TCA GAA AAT     1323
Arg Glu Asp Met Glu Arg Thr Cys Val Ile Pro Ser Ile Ser Glu Asn
        380                 385                 390

GAA CTT GAA GAT TTG GAA GAG GAA GCT AAA GAA GAA AAA TAT AAT GAT     1371
```

FIGURE 6 (CONT.)

```
GAC TCC TCC TAT ATA ATT GAA AGT GAT GAA GAT TTG GAA ATG GAG ATG      1467
Asp Ser Ser Tyr Ile Ile Glu Ser Asp Glu Asp Leu Glu Met Glu Met
                430                 435                 440

CTG AAG TCT TTA GAA AAC CTA AAT AGT GAC GTG GTG GAA CCC ACT CAC      1515
Leu Lys Ser Leu Glu Asn Leu Asn Ser Asp Val Val Glu Pro Thr His
            445                 450                 455

TCT ACA TGG TTG GAA ATG GGA ACC AAT GGG CGT CTT CCT CCT GAG GAG      1563
Ser Thr Trp Leu Glu Met Gly Thr Asn Gly Arg Leu Pro Pro Glu Glu
        460                 465                 470

GAA GAT GGA CAC GGA AAT GAA GCC ATC AAA GAG GAG CAG GAA GAA GAG      1611
Glu Asp Gly His Gly Asn Glu Ala Ile Lys Glu Glu Gln Glu Glu Glu
    475                 480                 485

GAC GAT TTA TTG CCG GAA CCC AAC GCA AAG CAA ATT AAT TGC CTC AAG      1659
Asp Asp Leu Leu Pro Glu Pro Asn Ala Lys Gln Ile Asn Cys Leu Lys
490                 495                 500                 505

ACC TAT TTC TGG GAC AGC AGT TTT AAA CCG GTT CAG TGG AAA GTC ATC      1707
Thr Tyr Phe Trp Asp Ser Ser Phe Lys Pro Val Gln Trp Lys Val Ile
                510                 515                 520

CAT TCT GTA TTA GAA GAG AGA AGA GAT AAT GTT GTT GTC ATG GCA ACT      1755
His Ser Val Leu Glu Glu Arg Arg Asp Asn Val Val Val Met Ala Thr
            525                 530                 535

GGA TAT GGG AAG AGT CTG TGC TTC ATG CAG TAT CCG CCT GTT TAT ACA      1803
Gly Tyr Gly Lys Ser Leu Cys Phe Met Gln Tyr Pro Pro Val Tyr Thr
        540                 545                 550

AAG ATT GGC ATT GTC ATT TCA CCT CTC ATT TCC TTA ATG GAA GAC CAA      1851
Lys Ile Gly Ile Val Ile Ser Pro Leu Ile Ser Leu Met Glu Asp Gln
    555                 560                 565

GTC GTG CAG TTT GAG CTG TCC AAT GTT GCA GCC TGT TTA GTT GGA TCT      1899
Val Leu Gln Phe Glu Leu Ser Asn Val Pro Ala Cys Leu Leu Gly Ser
570                 575                 580                 585

GCA CAG TCA AAA AAT ATT CTA GGA GAT GTT AAA TTA GGC AAA TAT AGG      1947
Ala Gln Ser Lys Asn Ile Leu Gly Asp Val Lys Leu Gly Lys Tyr Arg
                590                 595                 600

GTC ATC TAC ATA ACT CCA GAG TTC TGT TCT GGT AAC TTG GAT CTA CTC      1995
Val Ile Tyr Ile Thr Pro Glu Phe Cys Ser Gly Asn Leu Asp Leu Leu
            605                 610                 615

TAG GAA GTT GAG TGT AGT AAT CCC ATT ACT GTG ATT CCT CTA GAT GAG
```

FIGURE 6 (CONT.)

```
ATG CTG GGC TCT GTT AAA ACA GCG CTC CCA TTG GTT CCA GTC ATT GCA      2139
Met Leu Gly Ser Leu Lys Thr Ala Leu Pro Leu Val Pro Val Ile Ala
650                 655                 660                 665

CTC TCC GCT ACT GCA AGC TCT TCC ATC CGG GAA GAC ATT ATA AGC TGC      2187
Leu Ser Ala Thr Ala Ser Ser Ser Ile Arg Glu Asp Ile Ile Ser Cys
                670                 675                 680

TTA AAC CTG AAA GAC CCT CAG ATC ACC TGC ACT GGA TTT GAT CGG CCA      2235
Leu Asn Leu Lys Asp Pro Gln Ile Thr Cys Thr Gly Phe Asp Arg Pro
            685                 690                 695

AAT CTG TAC TTA GAA GTT GGA CGG AAA ACA GGG AAC ATC CTT CAG GAT      2283
Asn Leu Tyr Leu Glu Val Gly Arg Lys Thr Gly Asn Ile Leu Gln Asp
        700                 705                 710

TTA AAG CCA TTT CTA GTC CGA AAG GCA AGT TCT GCC TGG GAA TTT GAA      2331
Leu Lys Pro Phe Leu Val Arg Lys Ala Ser Ser Ala Trp Glu Phe Glu
    715                 720                 725

GGT CCA ACC ATC ATC TAT TGT CCT TCG AGA AAA ATG ACA GAA CAA GTT      2379
Gly Pro Thr Ile Ile Tyr Cys Pro Ser Arg Lys Met Thr Glu Gln Val
730                 735                 740                 745

ACT GTT GAA GTT GGG AAA CTG AAC TTA AAC TGC AGA ACA TAC GAC GCT      2427
Thr Val Glu Leu Gly Lys Leu Asn Leu Asn Cys Arg Thr Tyr Asp Ala
                750                 755                 760

GGC ATG AAA ATT AGC GAA AGG AAG GAC GTT CAT CAT AGG TTC CTG AGA      2475
Gly Met Lys Ile Ser Glu Arg Lys Asp Val His His Arg Phe Leu Arg
            765                 770                 775

GAT GAA ATT CAG TGT GTT GTA GCT ACT GTA GCT TTT GGA ATG GGC ATT      2523
Asp Glu Ile Gln Cys Val Val Ala Thr Val Ala Phe Gly Met Gly Ile
        780                 785                 790

AAT AAA GCT GAC ATT TGC AAA GTT ATT CAT TAT GGT GGG CCT AAG GTA      2571
Asn Lys Ala Asp Ile Arg Lys Val Ile His Tyr Gly Ala Pro Lys Glu
    795                 800                 805

ATG GAA TGC TAT TAC CAG GAA ATT GGT AGA GCT GGC CGG GAT GGA CTT      2619
Met Glu Ser Tyr Tyr Gln Glu Ile Gly Arg Ala Gly Arg Asp Gly Leu
810                 815                 820                 825

CAG AGT TGC TGT CAC TTG CTC TGG GCT CCA GCA GAC TTT AAC ACA TCC      2667
Gln Ser Ser Cys His Leu Leu Trp Ala Pro Ala Asp Phe Asn Thr Ser
                830                 835                 840

AGG AAT CTC CTT ACT GAG ATT GAA GAT GAA AAG TTC AGA TTA TAT AAA      2715
```

FIGURE 6 (CONT.)

```
AGG CGA CGA ATC ATC TTG TCC CAT TTT GAG GAC AAA TGT CTG CAG AAG      2811
Arg Arg Arg Ile Ile Leu Ser His Phe Glu Asp Lys Cys Leu Gln Lys
    875                 880                 885

GCC TCC TTG GAC ATT ATG GGA ACT GAA AAA TGC TGT GAT AAT TGC AGG      2859
Ala Ser Leu Asp Ile Met Gly Thr Glu Lys Cys Cys Asp Asn Cys Arg
890                 895                 900                 905

CCC AGG CTG AAT CAT TGC ATT ACT GCT AAC AAC TCA GAG GAC GCA TCC      2907
Pro Arg Leu Asn His Cys Ile Thr Ala Asn Asn Ser Glu Asp Ala Ser
                910                 915                 920

CAA GAC TTT GGG CCA CAA GCA TTC CAG CTA CTG TCT GCT GTG GAC ATC      2955
Gln Asp Phe Gly Pro Gln Ala Phe Gln Leu Leu Ser Ala Val Asp Ile
            925                 930                 935

CTG CAG GAG AAA TTT GGA ATT GGG ATT CCG ATC TTA TTT GTC CCA GGA      3003
Leu Gln Glu Lys Phe Gly Ile Gly Ile Pro Ile Leu Phe Val Pro Gly
        940                 945                 950

TCT AAT TCT CAG CGT CTT CCT GAT AAA TAT CGG GGT CAC AGG CTC TTT      3051
Ser Asn Ser Gln Arg Leu Pro Asp Lys Tyr Arg Gly His Arg Leu Phe
    955                 960                 965

GGT GCT GGA AAG GAG GAA CAA GTA GAA AGT TGG TGG AAG ACC CTT TCT      3099
Gly Ala Gly Lys Glu Glu Gln Val Glu Ser Trp Trp Lys Thr Leu Ser
970                 975                 980                 985

CAT CTC ATA GCT GAA GGA TTC TTG GTA CAA GTT CCC AAG GAA AAC AAA      3147
His Leu Ile Ala Glu Gly Phe Leu Val Gln Val Pro Lys Glu Asn Lys
                990                 995                1000

TAT ATA AAG ACA TGT TCC CTC ACA AAA AAG GGT AGA AAG TGG CTT GGA      3195
Tyr Ile Lys Thr Cys Ser Leu Thr Lys Lys Gly Arg Lys Trp Leu Gly
            1005                1010                1015

GAA GCC AGT TCG GAG TCT CCT CCG AGC CTT CTC CTT CAA GCT AAT GAA      3243
Glu Ala Ser Ser Glu Ser Pro Pro Ser Leu Leu Leu Gln Ala Asn Glu
        1020                1025                1030

GAG ATG TTT CCA AGG AAA GTT CTG CTA CCA AGT TCT AAT CCT GTA TCT      3291
Glu Met Phe Pro Arg Lys Val Leu Leu Pro Ser Ser Asn Pro Val Ser
    1035                1040                1045

CCA GAA ACG ACG CAA CAT TCC TCT AAT CAA AAC CCA GCT GGA TTA ACT      3339
Pro Glu Thr Thr Gln His Ser Ser Asn Gln Asn Pro Ala Gly Leu Thr
1050                1055                1060                1065

ACC AAG GAG TCT AAT TTG GAG AGA ACG CAT TCT TAC AAA GTG CCT GAG      3387
```

FIGURE 6 (CONT.)

```
TCA CCA GGA ACA TCT TCC AGC CCC TTA GAA CCT GCC ATC TCA GCC CAA      3483
Ser Pro Gly Thr Ser Ser Ser Pro Leu Glu Pro Ala Ile Ser Ala Gln
        1100                1105                1110

GAG CTG GAC GCT CGG ACT GGG CTA TAT GCC AGG CTG GTG GAA GCA AGG      3531
Glu Leu Asp Ala Arg Thr Gly Leu Tyr Ala Arg Leu Val Glu Ala Arg
    1115                1120                1125

CAG AAA CAC GCT AAT AAG ATG GAT GTA CCT CCA GCT ATT TTA GCA ACA      3579
Gln Lys His Ala Asn Lys Met Asp Val Pro Pro Ala Ile Leu Ala Thr
1130                1135                1140                1145

AAC AAG GTT CTG CTG GAC ATG GCT AAA ATG AGA CCC ACT ACT GTT GAA      3627
Asn Lys Val Leu Leu Asp Met Ala Lys Met Arg Pro Thr Thr Val Glu
                1150                1155                1160

AAC ATG AAA CAG ATC GAC GGT GTC TCT GAA GGC AAA GCT GCT CTG TTG      3675
Asn Met Lys Gln Ile Asp Gly Val Ser Glu Gly Lys Ala Ala Leu Leu
            1165                1170                1175

GCC CCT CTG TTG GAA GTC ATC AAA GAT TTC TGT CAA GTA ACT AGT GTT      3723
Ala Pro Leu Leu Glu Val Ile Lys His Phe Cys Gln Val Thr Ser Val
        1180                1185                1190

CAG ACA GAC CTC CTT TCC AGT GCC AAA CCT CAC AAG GAA CAG GAG AAA      3771
Gln Thr Asp Leu Leu Ser Ser Ala Lys Pro His Lys Glu Gln Glu Lys
    1195                1200                1205

AGT CAG GAG ATG GAA AAG AAA GAC TGC TCA CTC CCC CAG TCT GTG GCC      3819
Ser Gln Glu Met Glu Lys Lys Asp Cys Ser Leu Pro Gln Ser Val Ala
1210                1215                1220                1225

GTG AGA TAC ACT CTA TTC CAG GAA AAG AAA ATG CCC TTA CAC AGC ATA      3867
Val Thr Tyr Thr Leu Phe Gln Glu Lys Lys Met Pro Leu His Ser Ile
                1230                1235                1240

GCT GAG AAC AGG CTC CTG CCT CTC ACA GCA GCC GGC ATG CAC TTA GCC      3915
Ala Glu Asn Arg Leu Leu Pro Leu Thr Ala Ala Gly Met His Leu Ala
            1245                1250                1255

CAG GCC GTG AAA GCC GGC TAC CCC CTG GAT ATG GAG CGA GCT GGC CTG      3963
Gln Ala Val Lys Ala Gly Tyr Pro Leu Asp Met Glu Arg Ala Gly Leu
        1260                1265                1270

ACC CCA GAG ACT TGG AAG ATT ATT ATG GAT GTC ATC CGA AAC CCT CCC      4011
Thr Pro Glu Thr Trp Lys Ile Ile Met Asp Val Ile Arg Asn Pro Pro
    1275                1280                1285

ATG AAC TCA GAT ATG TAT AAA GTT AAA CTG ATC AGA ATG TTA GTT GCT      4059
```

FIGURE 6 (CONT.)

```
AGT GGT TCC GAC AGC AGA ACC CAG CCT CCT TGT GAT TCC AGC AGG AAG      4155
Ser Gly Ser Asp Ser Arg Thr Gln Pro Pro Cys Asp Ser Ser Arg Lys
            1325                1330                1335

AGG CGT TTC CCC AGC TCT GCA GAG AGT TGT GAG AGC TGT AAG GAG AGC      4203
Arg Arg Phe Pro Ser Ser Ala Glu Ser Cys Glu Ser Cys Lys Glu Ser
        1340                1345                1350

AAA GAG GCG GTC ACC GAG ACC AAG GCA TCA TCT TCA GAG TCA AAG AGA      4251
Lys Glu Ala Val Thr Glu Thr Lys Ala Ser Ser Ser Glu Ser Lys Arg
    1355                1360                1365

AAA TTA CCC GAG TGG TTT GCC AAA GGA AAT GTG CCC TCA GCT GAT ACC      4299
Lys Leu Pro Glu Trp Phe Ala Lys Gly Asn Val Pro Ser Ala Asp Thr
1370                1375                1380                1385

GGC AGC TCA TCA TCA ATG GCC AAG ACC AAA AAG AAA GGT CTC TTT AGT      4347
Gly Ser Ser Ser Ser Met Ala Lys Thr Lys Lys Lys Gly Leu Phe Ser
                1390                1395                1400

TAANATGACN ACGATTGAAC AGTTTGTGTG TCCTACATCT TCATTCCTAT AAAGAATGAA    4407

NAGAAATATT TTAACCTCAA AATTATTTAA AGTCCAAAGT GAAGCTCACC TAAACGTCGA    4467

GCCATAGAGT CTTTAATTGN CCGTTGGGAG TTGAGCTACA GTATCTGAAC CTTCTGAGAC    4527

CCGGAGTGCA GCATAGACTG TGAAGTCGGC TTCCTTTCCG ATTGCCTTCC GAACCCGTGT    4587

CACTGTCAGG TTGCAGTCTT TCTCTTCTTG CAGCAGTGTG TGTTGGAAAT GGAGGCTGTG    4647

TCGCTTTGAC ATATAGAACA GATCAGTAAT TGCATAGGGA CAGATATGAA GATNCAGCCG    4707

GTCTTTGCTT TCTTATCCAG ATGCCTCTAT GACAGTATCA GTGCACCAGC CCAGCCAGGG    4767

AGACATCAGC TTCCATTTAA AAAGG                                          4792
```

FIGURE 7

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

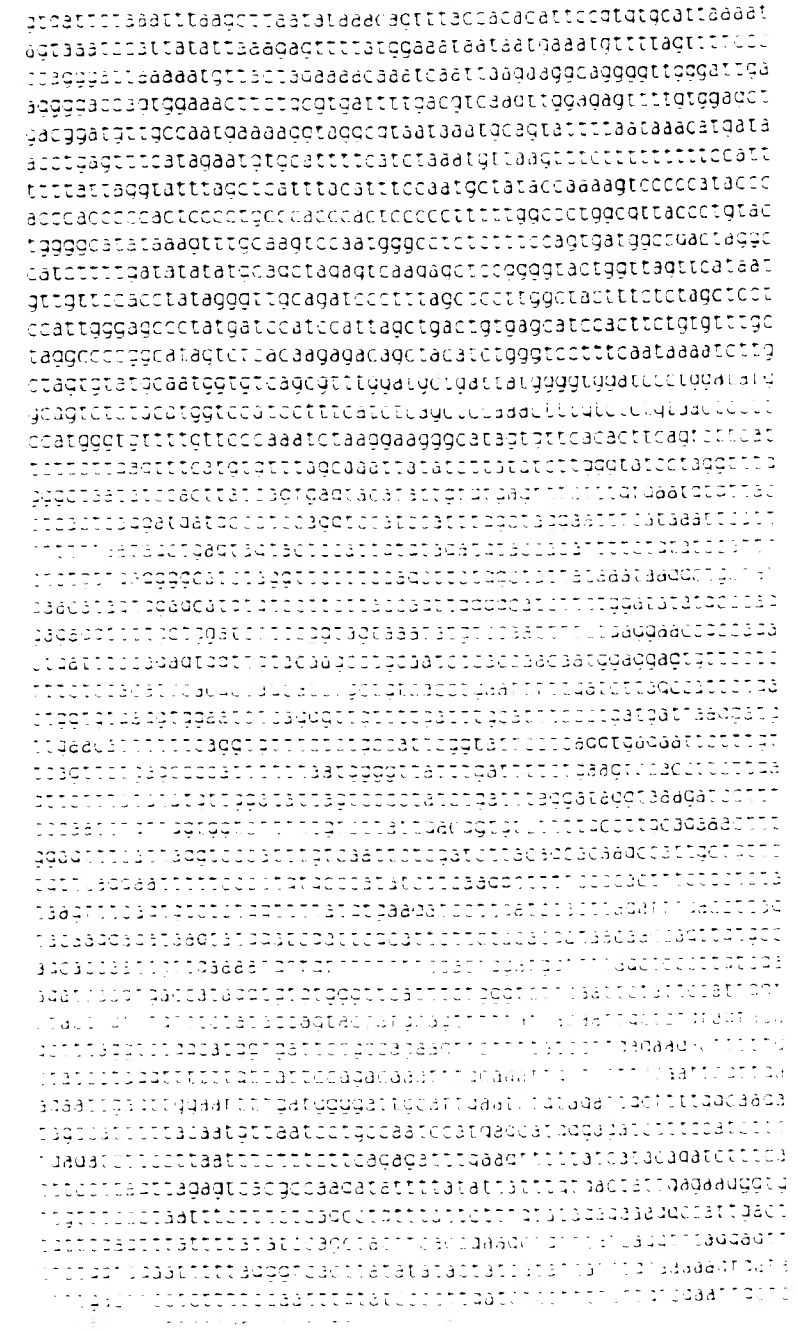


FIGURE 7 (CONT.)

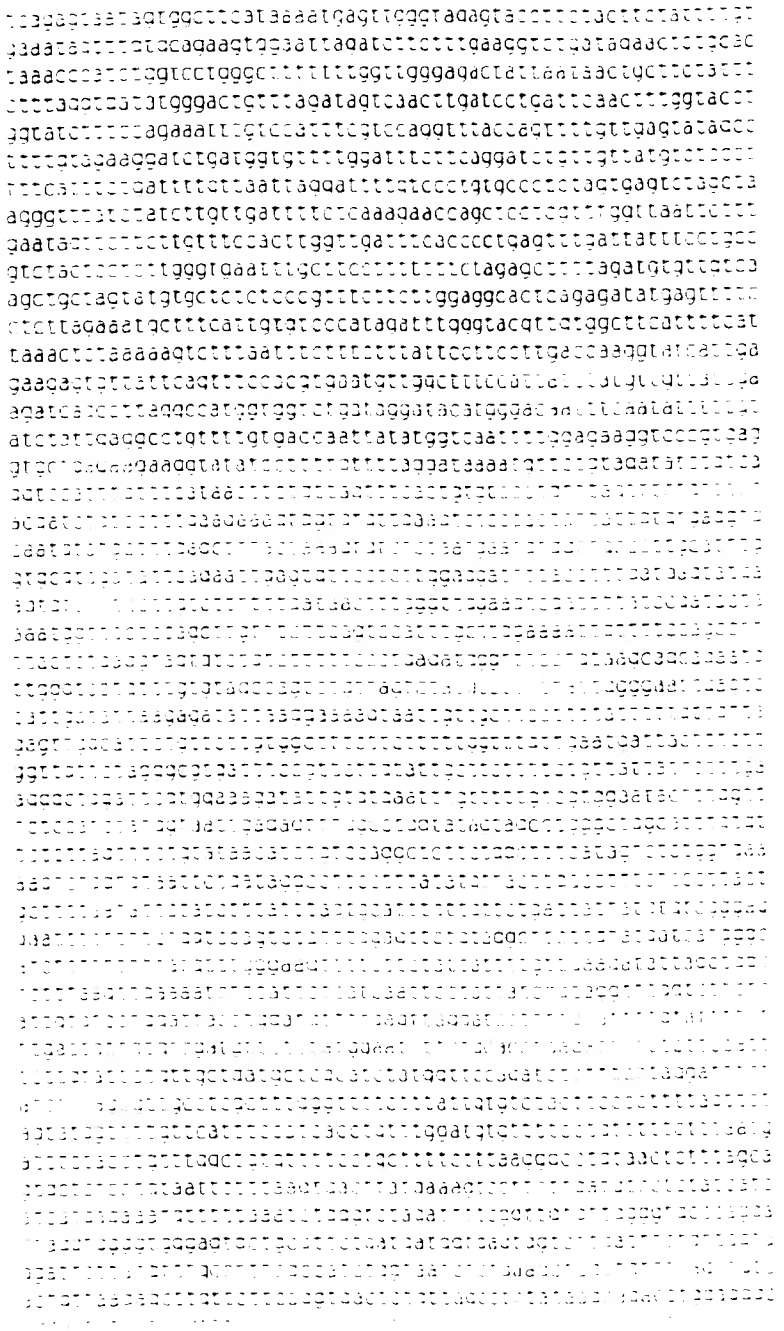


FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

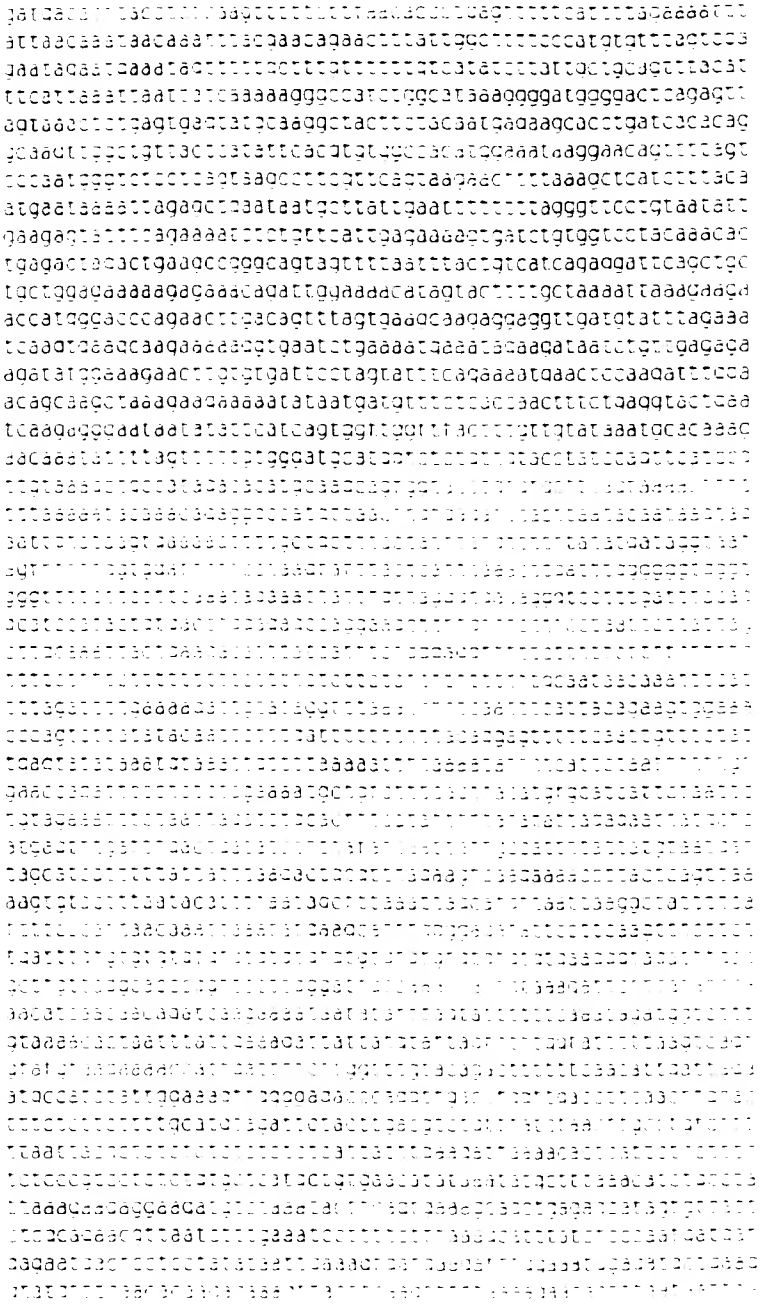


FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

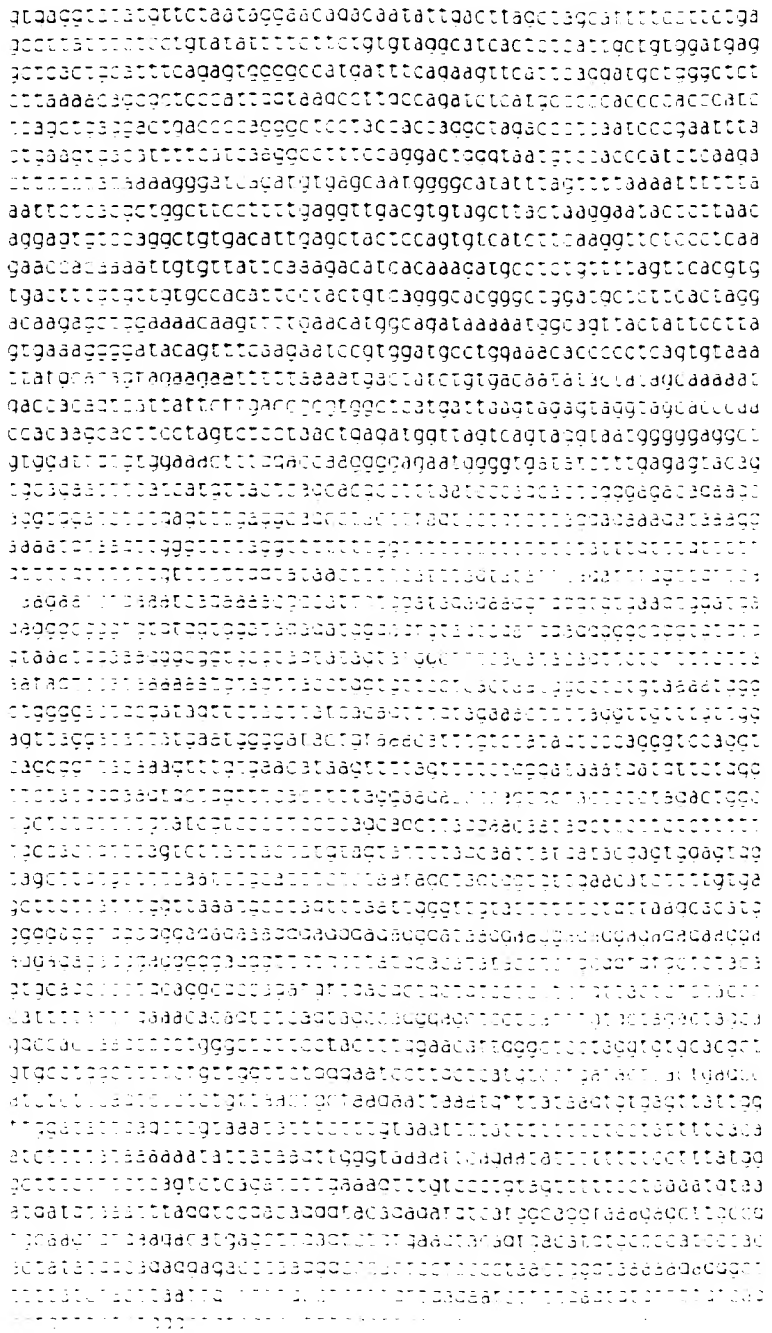


FIGURE 7 (CONT.)

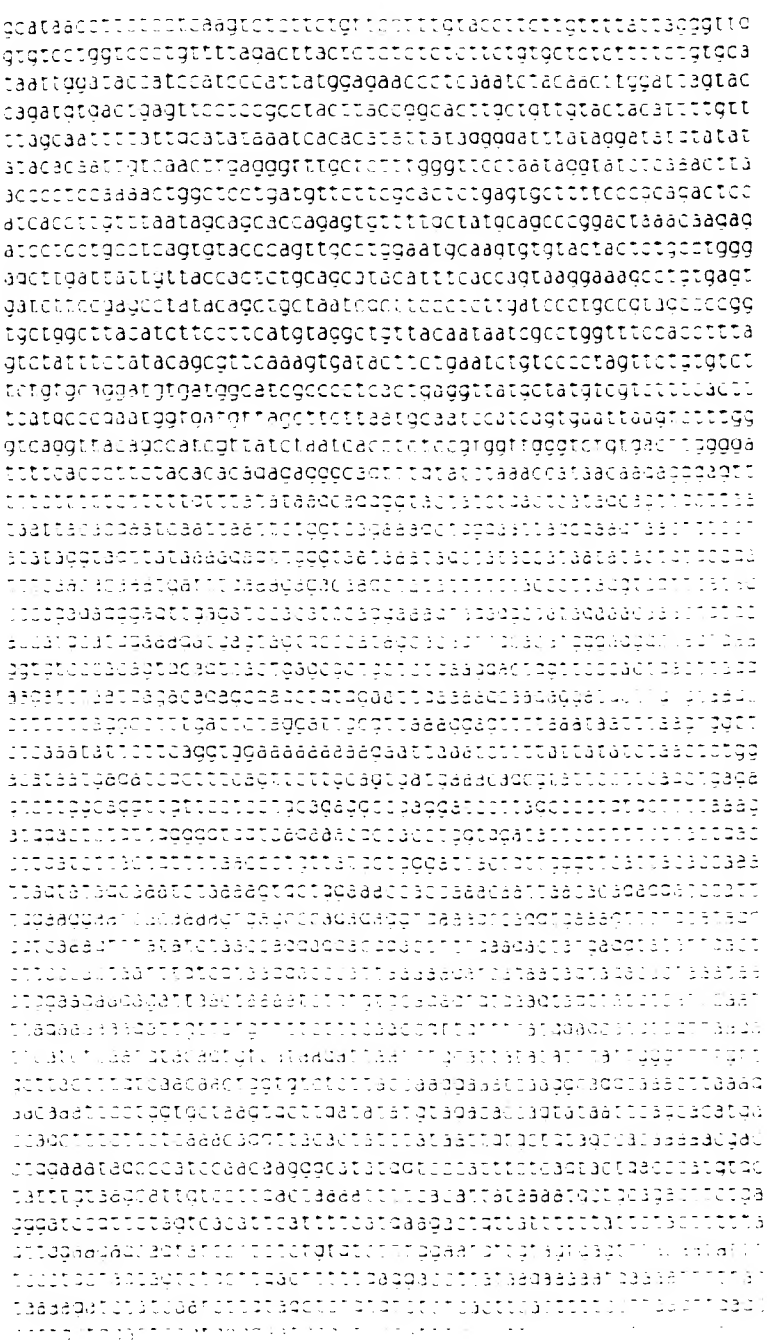


FIGURE 7 (CONT.)

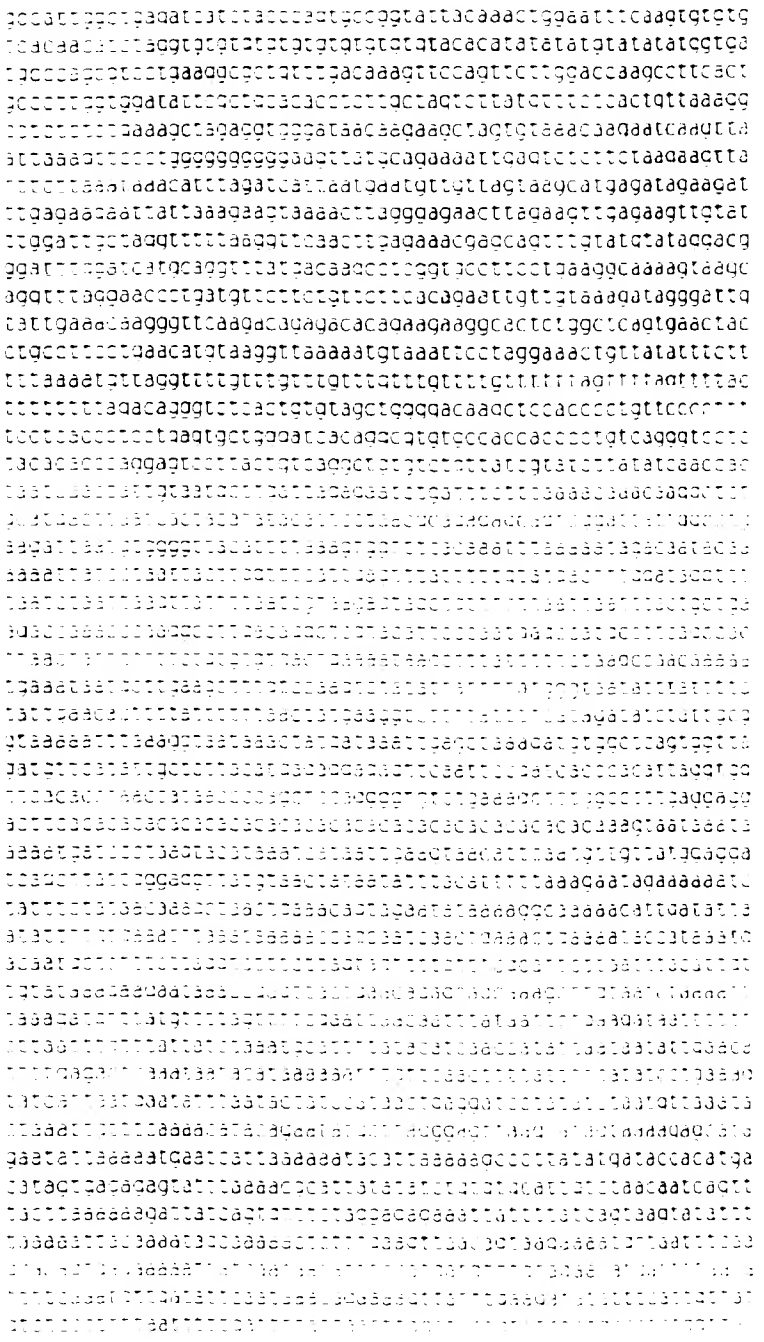


FIGURE 7 (CONT.)

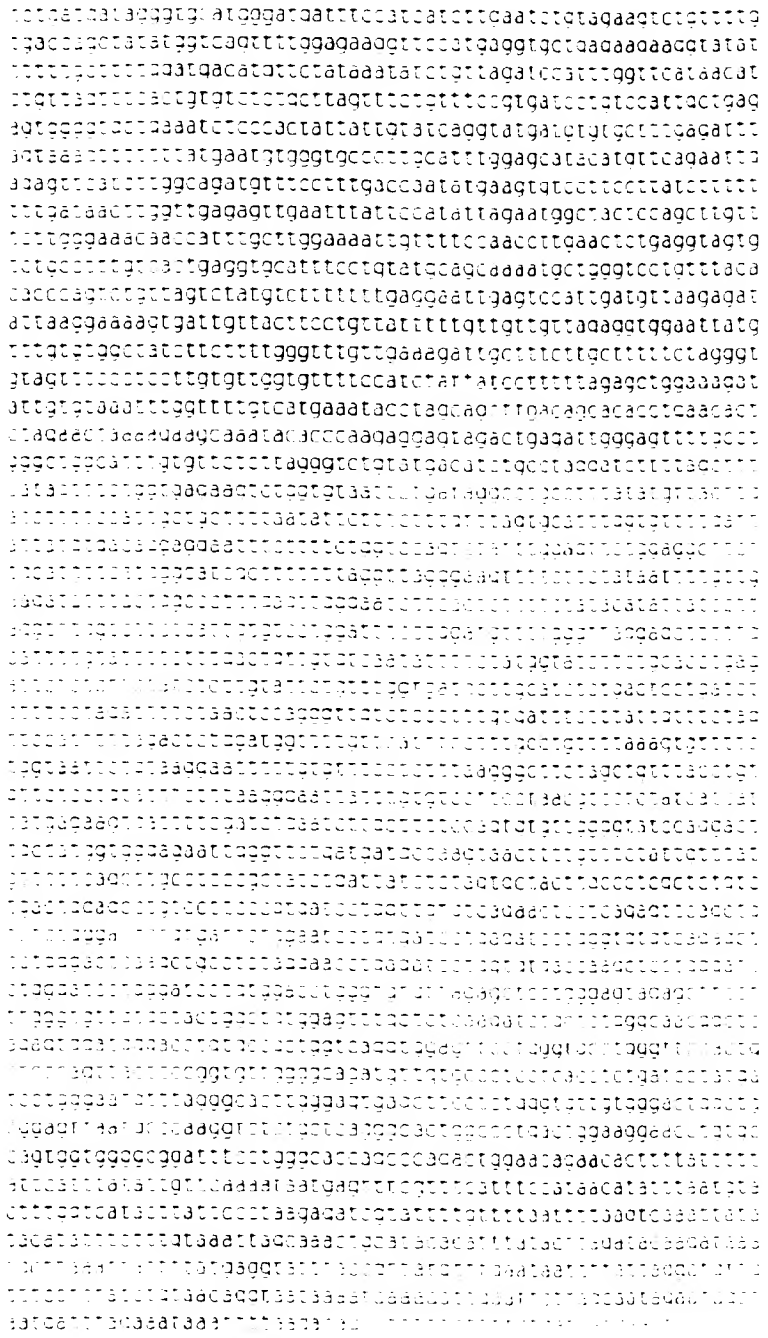


FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

FIGURE 7 (CONT.)

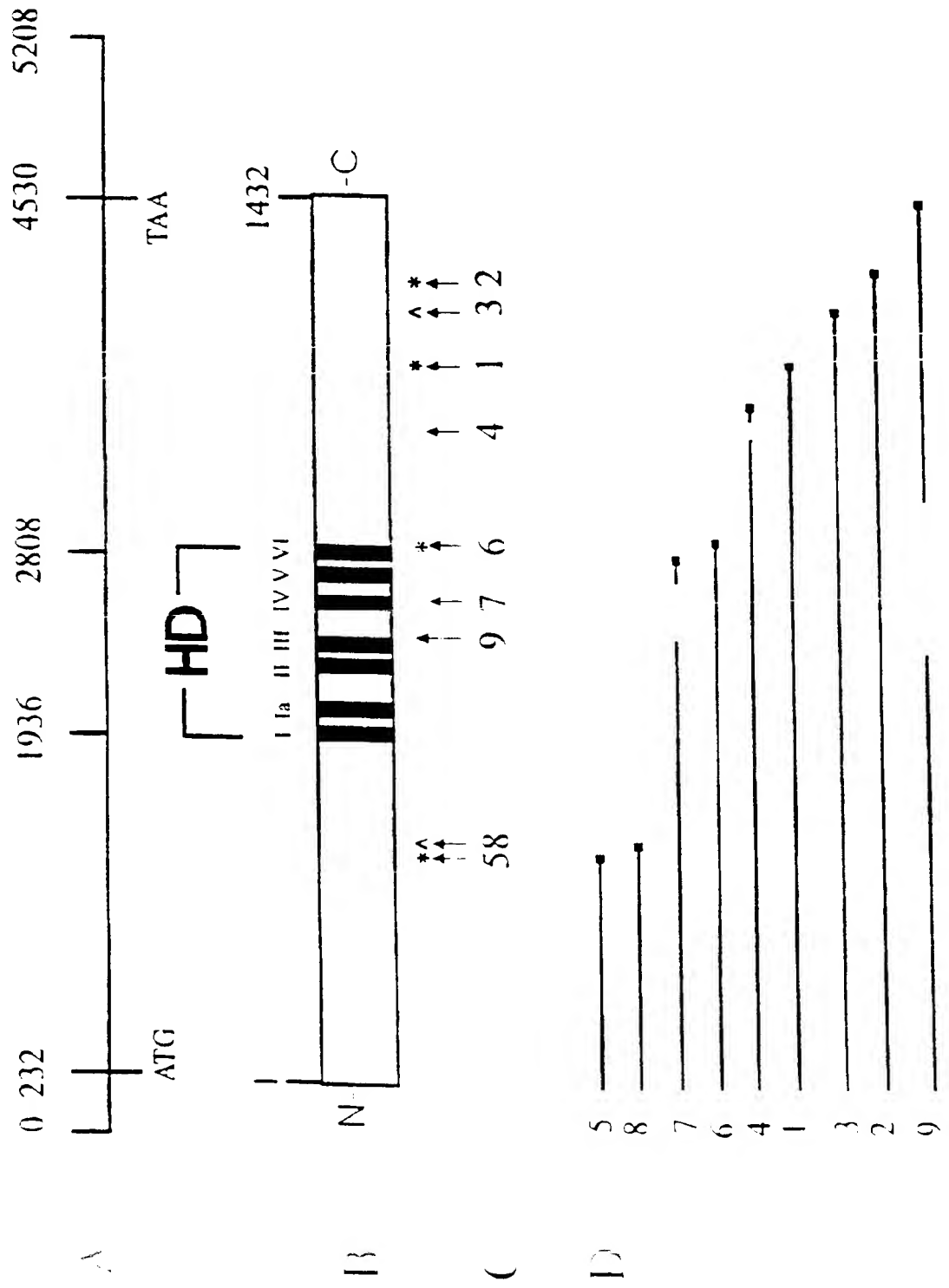

0 232
1936
2808
4530
5208
ATG
TAA
HD
I Ia II III IV V VI
1432
-C
N
58
9 7 6 4 1 3 2
5
8
7
6
4
1
3
2
9

FIGURE 8

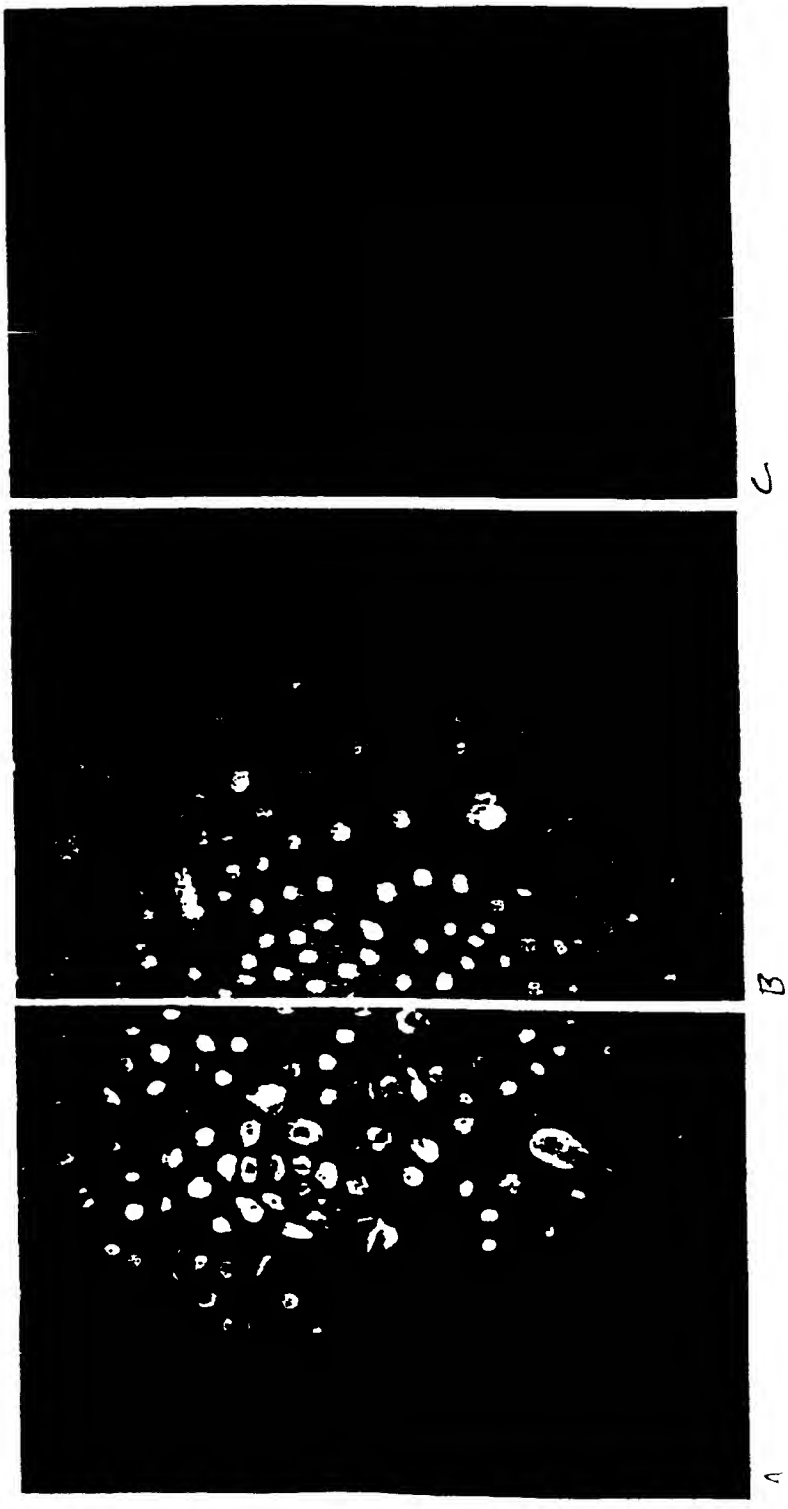


Figure 9. Detection of the WRN protein by indirect immunofluorescence in adherent human epithelial cells. Cells from prostate epithelium were placed on a glass slide and allowed to grow for several days before staining. The cells were fixed, and stained simultaneously for the WRN protein, DNA in the nucleus, or F-actin in the cytoplasm. Panel A was after staining with an appropriate dilution of the anti-WRN peptide rabbit antiserum. The second by antibody goat tagged with FITC. Panel B fluorescence on the near ultra violet after staining nuclear DNA with bis benzimidine. Panel C was arising from F-actin stained with BODIPY tagged phalloidin. 200X magnification

FIGURES 9A, B and C

FIGURE 10

Werne Human-Mouse Annotated Protein Alignment

[illegible] exons 10 & 11
GREEN - Indic[illegible] splits
Download seq files: Human or Mouse

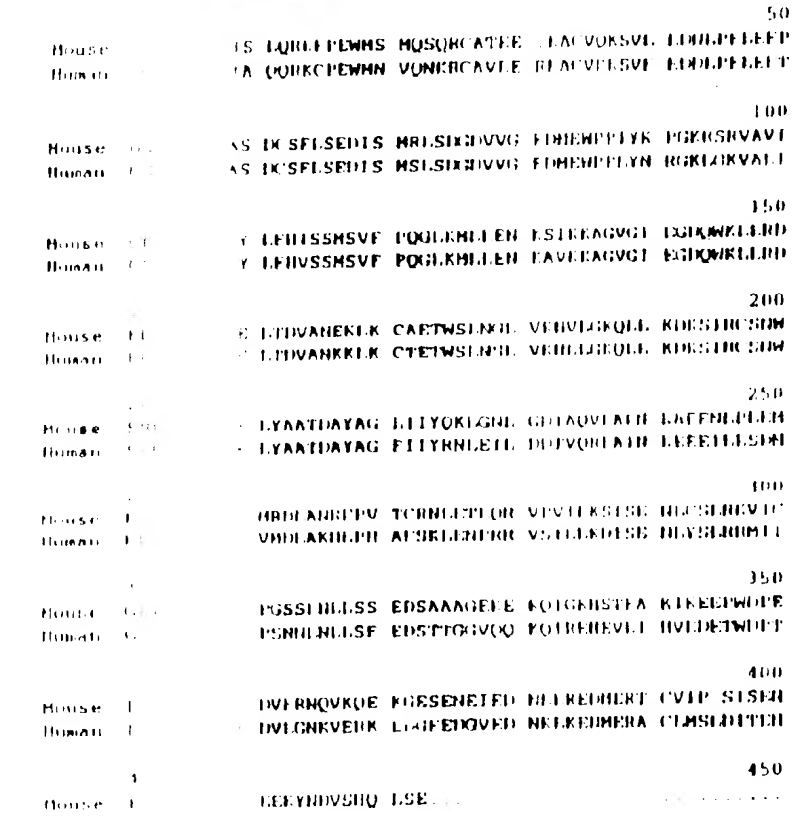


FIGURE 10 (CONTINUED)

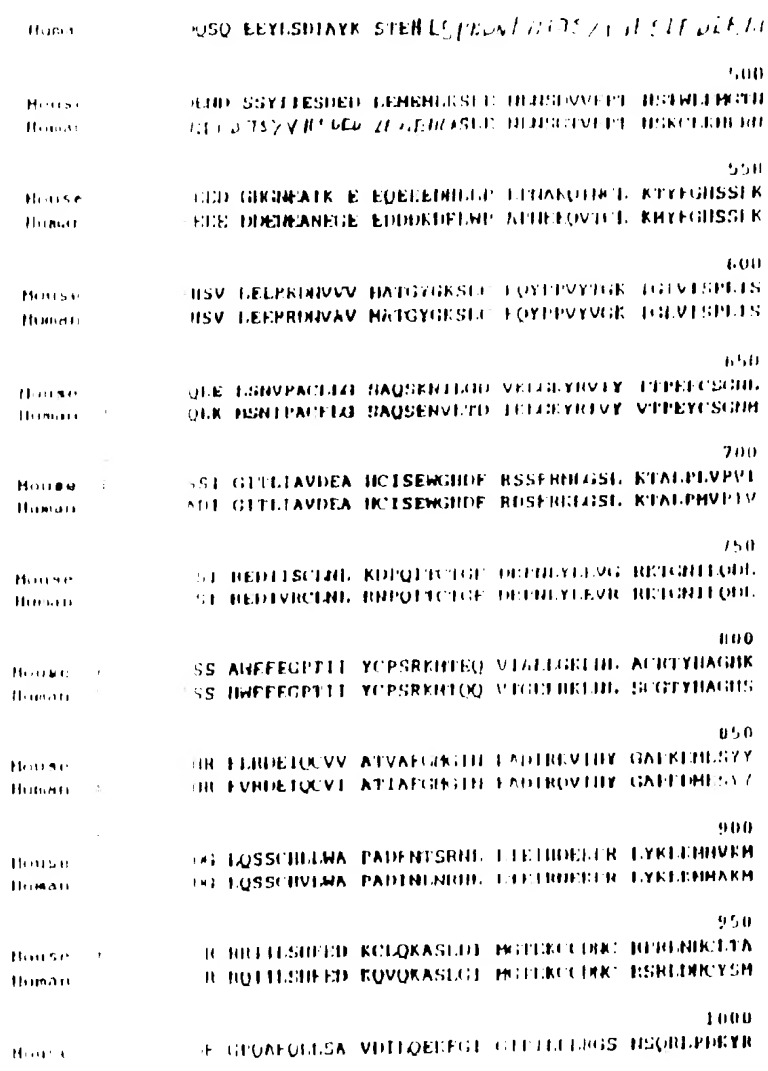
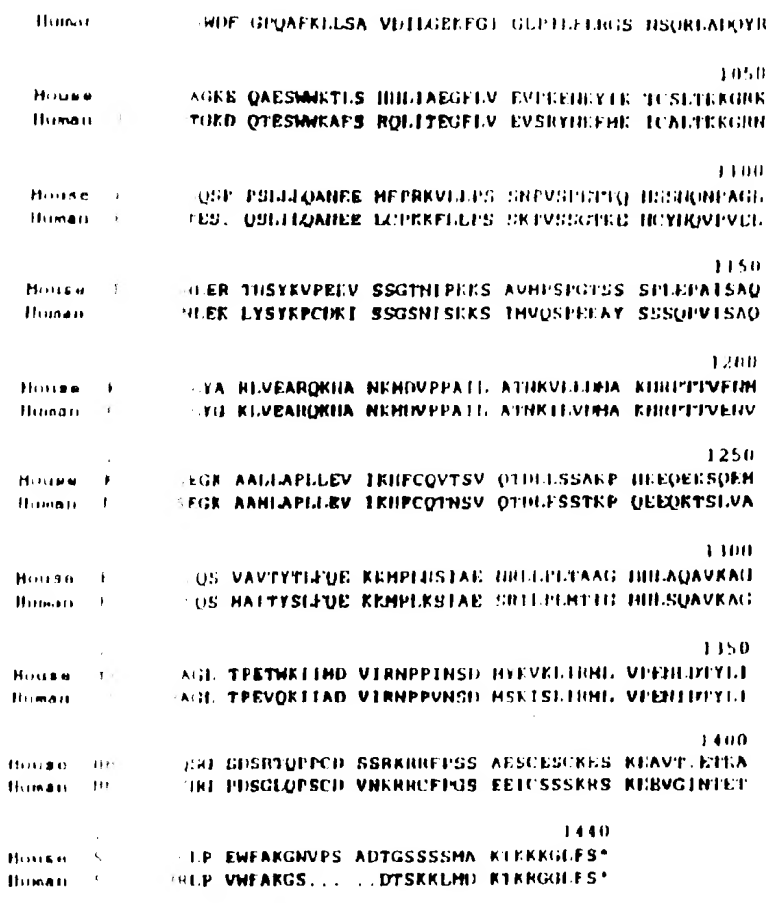


FIGURE 10 (CONTINUED)



Analysis and w[illegible] generated by A. Swartz
Last Update [illegible]

INTERNATIONAL SEARCH REPORT

Ir ational Application No
PCT/US 96/20785

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/00 C12N15/61 C12N9/90 C12N15/85 C12N15/86
C12N5/10 C12N5/20 C07K16/40 C12Q1/68 A01K67/027
G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL SEQUENCE DATABASE, 30 January 1995, HEIDELBERG, BRD, XP002032088 F. BOUILLAUD: "Study of expressed sequence tags in adipose tissue 1995" accession no. T39125 see abstract ---	2,15,16
X	EMBL SEQUENCE DATABASE, 29 May 1995, HEIDELBERG, BRD, XP002032089 R. BERRY ET AL.: "Gene-based sequence tagged sites (STSs) as the basis for a human gene map" see accession no. R58879 see abstract --- -/--	2,15,16

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 June 1997	10.06.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040 Fax (+31-70) 340-3016	

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir ational Application No
PCT/US 96/20785

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol. 355, 20 February 1992, MACMILLAN JOURNALS LTD., LONDON,UK, pages 735-738, XP002032090 M. GOTO ET AL.: "Genetic linkage of Werner's syndrome to five markers on chromosome 8" cited in the application see the whole document	1-21
A	AMERICAN JOURNAL OF HUMAN GENETICS 55 (2). 1994. 356-364. ISSN: 0002-9297, August 1994, XP000674409 YU C-E ET AL: "Linkage disequilibrium and haplotype studies of chromosome 8p 11.1-2.1 markers and Werner syndrome." cited in the application see the whole document	1-21
A	ANNUAL MEETING OF THE AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, SAN FRANCISCO, CALIFORNIA, USA, MAY 21-25, 1995. FASEB JOURNAL 9 (6). 1995. A1270. ISSN: 0892-6638, 24 April 1995, XP002032091 THWEATT R ET AL: "A novel cDNA overexpressed in Werner syndrome (WS) fibroblasts inhibits colony formation in normal human fibroblasts and in HeLa cells." abstract no.84 see abstract	1-21
A	GENOMICS 28 (2). 1995. 147-153. ISSN: 0888-7543, 20 July 1995, XP000674353 KURIMASA A ET AL: "Construction of 110 cosmid markers and a 4.5-Mb YAC contig on human chromosome 8p12-q11." see the whole document	1-21
A	GENOMICS 28 (3). 1995. 566-569. ISSN: 0888-7543, 10 August 1995, XP000674354 YE L ET AL: "Genetic association between chromosome 8 microsatellite (MS8-134) and Werner Syndrome (WRN): Chromosome	1-21

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/20785

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	45TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HUMAN GENETICS, MINNEAPOLIS, MINNESOTA, USA, OCTOBER 24-28, 1995. AMERICAN JOURNAL OF HUMAN GENETICS 57 (4 SUPPL.). 1995. A266. ISSN: 0002-9297, October 1995, XP000674410 NAKURA J ET AL: "Genetic association between chromosome 8 microsatellites and Werner syndrome (WRN)." abstract no. 1544 see abstract ---	1-21
A	NUCLEIC ACIDS RESEARCH, vol. 22, no. 22, 11 November 1994, IRL PRESS LIMITED.OXFORD.ENGLAND, pages 4566-4573, XP002032092 M. SEKI ET AL.: "Molecular cloning of cDNA encoding human DNA helicase Q1 which has homology to Escherichia coli Rec Q helicase and localization of the gene at chromosome 12p12" see the whole document ---	1-21
A	J. BIOL. CHEM., vol. 269, no. 47, 25 November 1994, AM. SOC. BIOCHEM. MOL.BIOL.,INC.,BALTIMORE,US, pages 29838-29845, XP002032093 K.L. PURANAM AND P.J. BLACKSHEAR: "Cloning and characterization of RECQL, a potential human homologue of the Escherichia coli DNA helicase RecQ" see the whole document ---	1-21
P,X	SCIENCE (WASHINGTON D C) 272 (5259). 1996. 258-262. ISSN: 0036-8075, XP002032094 YU C-E ET AL: "Positional cloning of the Werner's syndrome gene." see the whole document ---	1,2,10
P,X	TRENDS IN GENETICS 12 (8). 1996. 283-286. ISSN: 0168-9525, August 1996, XP002032095 LOMBARD D B ET AL: "Cloning the gene for Werner syndrome: A disease with many symptoms of premature aging." see the whole document ---	1,2,10, 15,16
P,X	HUMAN MOLECULAR GENETICS 5 (12). 1996 [illegible] heterozygous [illegible] WRN syndrome locus. see the whole document ---	1,2,10

-/--

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/20785

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	AMERICAN JOURNAL OF HUMAN GENETICS 60 (2). 1997. 330-341. ISSN: 0002-9297, February 1997, XP000674411 YU C-E ET AL: "Mutations in the consensus helicase domains of the Werner syndrome gene." see the whole document ---	1-21
T	GENOMICS 41 (2). 1997. 298-300. ISSN: 0888-7543, XP000674356 IMAMURA O ET AL: "Cloning of a mouse homologue of the human Werner syndrome gene and assignment to 8A4 by fluorescence in situ hybridization." see the whole document -----	1-21

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US95/05330

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; C12N 5/00, 5/06, 5/08, 5/10, 5/12, 5/16, 5/22

US CL :435/240.1, 240.2, 172.3, 320.1; 424/93.21; 935/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.1, 240.2, 172.3, 320.1; 424/93.21; 935/62

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, APS, bystander#, migration, msc, mc, gene#, dna#, cdna#, rna#, mrna#. gene, thera?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93/02556 [FREEMAN ET AL] 18 Februray 1993, see entire document.	1-33
Y	Science, Volume 256, issued 12 June 1992, K.W. Culver, "In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors", pages 1550-1552, see entire document.	1-33
Y	Cancer Research, Volume 53, issued 01 January 1993, Z. Ram et al, "In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats", pages 83-88, see entire document.	1-33

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international [illegible]

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" [illegible]

[illegible] considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

24 JULY 1995

Date of mailing of the international search report

03 AUG 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JAMES MARTINELL

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US95/05330

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, USA, Volume 85, issued October 1988, E. Borrelli et al, "Targeting of an inducible toxic phenotype in animal cells", pages 7572-7576, see entire document.	1-33
Y	Journal of Experimental Medicine, Volume 172, issued October 1990, B. Gansbacher et al, "Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity", pages 1217-1224, see entire document.	1-33
Y	Science, Volume 254, issued 01 November 1991, P.T. Golumbek et al, "Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4", pages 713-716, see entire document.	1-33
Y	Journal of Experimental Medicine, Volume 177, issued May 1993, L.E. Minasi et al, "The selective ablation of interleukin 2-producing cells isolated from transgenic mice", pages 1451-1459, see entire document.	1-33